A Study of the Constituents of the Brown Marine Algae

by

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Thesis for the Degree of D.Sc.

1956

January, 1956.

CONTENTS

Synopsis

Although in the last 250 years seaweeds have been used on an industrial scale, first as a source of alkali for the soap and glass industries, then as a source of iodine, and latterly for the production of alginic acid, practically nothing was known of their chemical composition and the effect of season and other influencing factors on their composition.

In 1944 the Scottish Seaweed Research Association was formed with the object of (1) surveying the coast of Scotland to ascertain if there was sufficient seaweed to justify the setting up of a large scale industry, (2) studying the factors which influenced composition and (3) devising economical means of harvesting seaweed.

The surveys showed that only the brown seaweeds (Phaeophyceae) occurred in sufficient quantity to warrant further investigation. Consequently, a study was commenced on the factors likely to influence their chemical composition.

Factors affecting the Chemical Composition

The effect of season and degree of exposure was first examined for the three most commonly occurring sublittoral species[1]. For a period of two years monthly samples of the Laminariaceae, Laminaria cloustoni, L. digitata and L. saccharina, were collected, with the L. digitata and the L. saccharina samples taken from two different habitats to examine the effect of degree of exposure on chemical composition. The samples were first analysed for moisture, total ash, iodine/...

iodine, Kjeldahl nitrogen, mannitol, laminarin and alginic acid and later for cellulose[2] and combined L-fucose[3]. This work was continued for a further two years and, in addition, the seasonal variation in the wet weight of the plants was studied[4]. At the same time samples of seawater were taken from above the weed beds and the chemical composition of the seaweeds correlated with that of the seawater in which they were growing[5].

The effect of depth of immersion on the chemical composition of the Laminariaceae was also studied[6].

Monthly samples of the common littoral seaweeds, Fucus serratus, F. vesiculosus, F. spiralis, Pelvetia canaliculata and Ascophyllum nodosum were also taken, with A. nodosum collected from three different habitats to determine the effect of degree of exposure on chemical composition[7,8,2,3].

A study was carried out on the concentration gradients in Laminaria saccharina fronds and it was shown that they do exist at certain times of the year, contrary to the belief that such gradients are not required in seaweeds of this type for the translocation of matter[9].

Isolation of Chemicals from the Brown Marine Algae

As the seaweed industry in Britain is based solely on the extraction of alginic acid, methods were worked out for the isolation of the other constituents as by-products of the alginate process[10,11,12]. Employing these methods, quantities of mannitol, laminarin and fucoidin were isolated and purified for studies which were being carried out by collaborative/...

collaborative investigators at a number of universities.

Laminarin and fucoidin were also considered as sources of D-glucose and L-fucose respectively, and the optimum conditions for the isolation of these sugars worked out[13,14]. In view of an increasing demand for the sugar D-galactose, different species of red seaweeds were examined as possible sources, as well as indigenous plant material such as bracken, heather, Sphagnum moss and peat[15].

In view of the commercial importance of alginic acid and the belief that it could only be satisfactorily obtained from Laminaria cloustoni stipes, an investigation was carried out to prove that it can be isolated from any of the common brown seaweeds; variations did occur, however, in the degree of polymerisation of the acids isolated from different species[16].

As a result of the success of initial trials with sodium laminarin sulphate as a blood anticoagulant, its preparation and that of other possibly useful derivatives of laminarin were studied[17].

The use of charcoal columns in separating mixtures of salts, mannitol, D-glucose and L-fucose, such as are obtained in seaweed hydrolysates, was examined. The disaccharide, laminaribiose, was isolated from a partial hydrolysate of laminarin on charcoal[18].

The possibility of preparing sex hormones from fucosterol, which occurs in the brown seaweeds, was considered and a method for its determination worked out and applied to samples of seaweed[19].

The Preservation of Seaweeds

As/...

As the chemicals already discussed could probably be isolated more cheaply from fresh seaweed, without the additional cost of drying, methods of preserving fresh samples were studied and the chemical changes which occur on ensiling were investigated[20].

A recent review of the above work and that carried out by other investigators in this field was published in the Annual Reports of the Chemical Society for 1953[21].

In a number of the publications the writer's name appears in conjunction with other workers but in all these cases he was the sole director of the work.

THE SCOTTISH SEAWEED RESEARCH ASSOCIATION

TECHNICAL REPORT

REFERENCE C/2

The Seasonal Variation in Chemical Constitution of some of the Sub-Littoral Seaweeds Common to Scotland

Part I. Laminaria Cloustoni.
Part II. Laminaria digitata.
Part III. Laminaria saccharina and Saccorhiza bulbosa.

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Reprinted from
THE JOURNAL OF THE SOCIETY OF CHEMICAL INDUSTRY, 1948
67, *pp. 165–168 : 169–172 : 172–176*

THE SCOTTISH SEAWEED RESEARCH ASSOCIATION
INVERESK GATE, MUSSELBURGH, MIDLOTHIAN
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Reprinted from the Journal of the Society of Chemical Industry, 1948, **67,**
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THE SEASONAL VARIATION IN CHEMICAL CONSTITUTION OF SOME OF THE SUB-LITTORAL SEAWEEDS COMMON TO SCOTLAND

PART I. *LAMINARIA CLOUSTONI*
PART II. *LAMINARIA DIGITATA*
PART III. *LAMINARIA SACCHARINA* AND *SACCORHIZA BULBOSA*

By W. A. P. Black

PART I. *LAMINARIA CLOUSTONI*

It is well known that seaweed, vast quantities of which grow around the coast of Scotland, undergoes wide seasonal variation in chemical constitution, and it is important that this variation be known so that the weed can be harvested at the appropriate period. Monthly samples of *Laminaria cloustoni* have been collected at a definite spot and at a constant depth over a period of two years, the plants divided into stipe and frond and analysed separately for total ash, organic nitrogen, iodine, mannitol, laminarin and alginic acid. The results show that the wide seasonal variations in composition, previously recorded, are due almost entirely to variations in the composition of the fronds. Samples have also been taken at depths of ½ to 6½ fathoms (L.W.) and the variation in composition with depth of immersion studied.

Although fairly large quantities of seaweed are available around the coast of Scotland, very little use is at present being made of it as a source of raw material. This may be due to the fact that it has not been sufficiently studied in this country from the chemical point of view and no reliable analytical methods have been available for the estimation of its constituents.

A complete review of all the literature on this subject, scattered through botanical, agricultural, chemical and physical journals, appears almost impossible. It shows many contradictions and indicates that in practically every country where seaweed is available, investigations have been carried out to show its importance as a source of raw material. These investigations, however, have rarely been of a chemical nature. Considerable progress has certainly been made from the botanical point of view, but

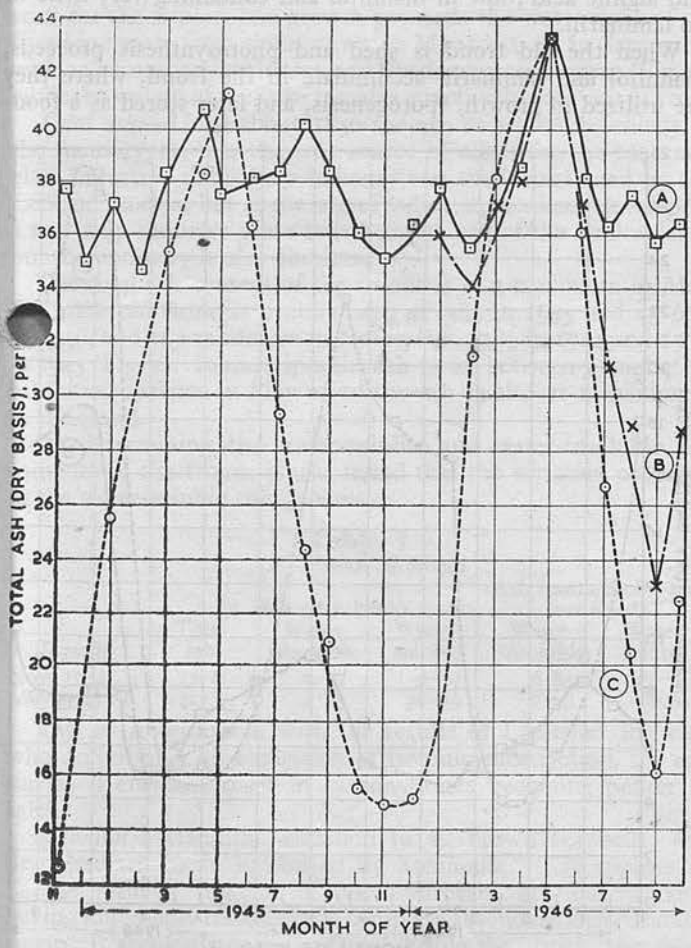

FIG. 1. *Seasonal variation in total ash*
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

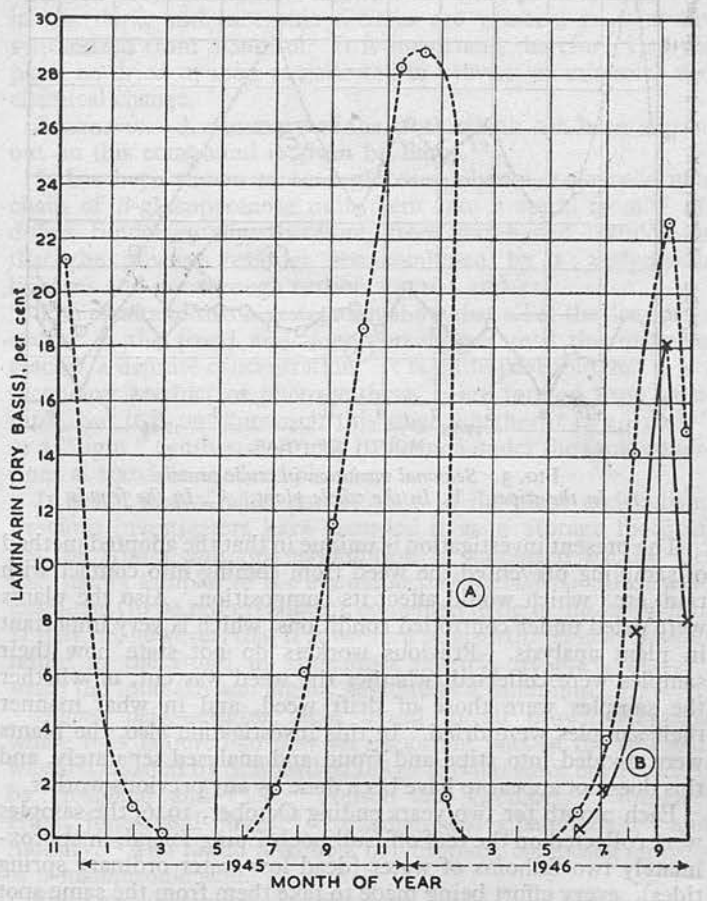

FIG. 2. *Seasonal variation in laminarin*
A. *In the frond* B. *In the whole plant*

very little is known about the seasonal variation in chemical constitution of the different weeds, a variation which greatly affects the value of the weed as a raw material. As any future industry based on seaweed will most likely depend on its organic constituents which are mainly polysaccharide in character, a knowledge of the seasonal variation is essential so that the weed can be harvested at the appropriate period.

Many reviews have been given of Stanford's work[1] in the second half of the last century. He laid the foundations for an industry based on the organic constituents of seaweed and his work will remain a classic for other workers in this field.

During the first world war the demand for potash led to an extensive investigation being carried out by Hendrick[2] but he was chiefly concerned with the analysis of the mineral matter of the weeds.

In 1919 Lapicque[3] carried out the first systematic investigation into the seasonal variation of the marine algae and his results showed that the phenomenon conformed to the laws of physiology. The work was continued by Colin and Ricard[4] who collected monthly samples of *L. flexicaulis* and *L. saccharina* during 1927–1929 and examined them for dry weight, ash, mannitol, laminarin, algin and cellulose. In 1935 Lunde,[5] investigating the possibilities of the seaweed off the coast of Norway, as a source of raw material, collected monthly samples of *L. digitata* during two years and analysed them for ash, mannitol, alginic acid, laminarin, fucoidin and nitrogen.

Similar investigations have been carried out in Russia,[6] Japan,[7] and Eire.[8]

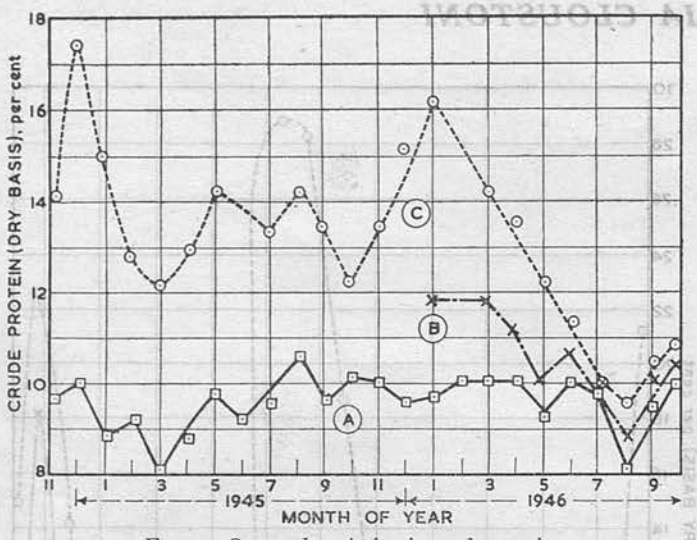

FIG. 3. *Seasonal variation in crude protein*
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

The present investigation is unique in that the adopted method of sampling prevented the weed from coming into contact with rain, etc., which would affect its composition. Also the plants were dried under controlled conditions, which is very important in plant analysis. Previous workers do not state how their samples were collected, whether the weed was cut, or whether the samples were those of drift weed, and in what manner their samples were dried. In this investigation also, the plants were divided into stipe and frond and analysed separately, and this does not appear to have been done by any previous worker.

Each month for two years ending October, 1946, the samples were collected on the reef off Cullipool, Luing Island, in approximately two fathoms of water (dead low water ordinary spring tides), every effort being made to take them from the same spot and at the same depth. The samples were taken by trawling a multi-pronged grapnel for two minutes, twisting the grapnel and lifting the weed into the boat. The depth samples were taken off the Island of Shapinsay, in the Orkneys, by means of a spring grab, which closed by trigger action on striking the bottom, cutting and enclosing all the weed within an area of $\frac{1}{2}$ square yard. Two plants were picked at random, and after being separated into stipes and fronds were draped over racks in a heated shed and dried at a temperature of 25–35° for 48 hours, after which they were ground in a Christy & Norris No. 8 Laboratory Mill, fitted with a $\frac{1}{64}$ in. perforated plate screen, giving a powder which practically all passed 90 mesh. As the analytical methods were evolved it was found that this fine state of division was essential, especially for the method of estimating alginic acid.

To ensure that the method of sampling adopted was not open to objection, individual plants were collected at the same time and place, and analysed. The analytical results obtained agreed very closely and indicated that the method of sampling the weed was relatively sound.

Experimental

Dry weight content.—This was determined by taking samples from three sections of the stipe and frond, and drying them to constant weight in an oven at 100°.

Moisture content of milled samples.—Determined by drying a 5 gram sample in an oven at 100° for 6 hours.

Total ash content.—The method of dry ashing was adopted, 1·5–2·0 g. of the sample were heated at dull redness to constant weight in a porcelain crucible.

Insoluble ash content.—1·5 g. of weed were heated in a porcelain crucible at dull redness for 30–45 minutes, allowed to cool, and extracted with 4 × 5 ml. of hot water, decanting off th clear liquid, after each extraction through a 9 cm. ashless filte paper and washing the bulk of the solid on to the paper with th last treatment. The solid on the filter paper was washed wit 4 × 5 ml. of boiling water, and the paper transferred back to th crucible and ashed to constant weight.

Iodine content.—By the method of Baggesguard-Rasmusse

Crude protein.—The total organic nitrogen was determin by the standard Kjeldahl method and multiplied by 6·25.

Mannitol, laminarin and alginic acid.—By the methods reporte by Cameron, Ross and Percival.[10]

Discussion of results

Seasonal variation in composition at constant depth

General.—The results obtained are all calculated on th anhydrous basis and are summarized in the graphs. In 194 the fronds and stipes were weighed separately, enabling th results to be calculated for the whole plant. In general the curv for the stipe, frond and whole plant coincide in the spring a then as the dry weight content increases in the frond only (Fig. the curve for the whole plant follows that of the frond. It w be noted that the marked seasonal variation in chemical compos tion occurs mainly in the fronds, where most, if not all of th photosynthesis occurs, while the stipes show only slight var ations. This is logical when it is considered that the frond an annual formation while the stipe is perennial. In the sprin when the new frond is forming and before photosynthes commences to any degree, the composition of the frond similar to that of the stipe, i.e., high in water, mineral matt and alginic acid; low in mannitol and containing very little no laminarin.

When the old frond is shed and photosynthesis proceed mannitol and laminarin accumulate in the frond, where th are utilized in growth, sporogenesis, and later stored as a foo

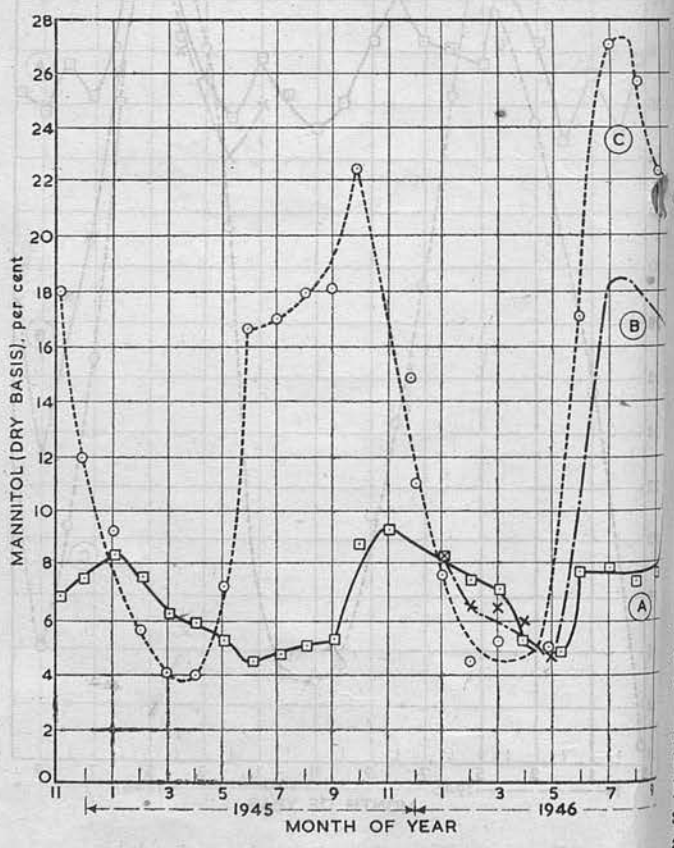

FIG. 4. *Seasonal variation in mannitol*
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

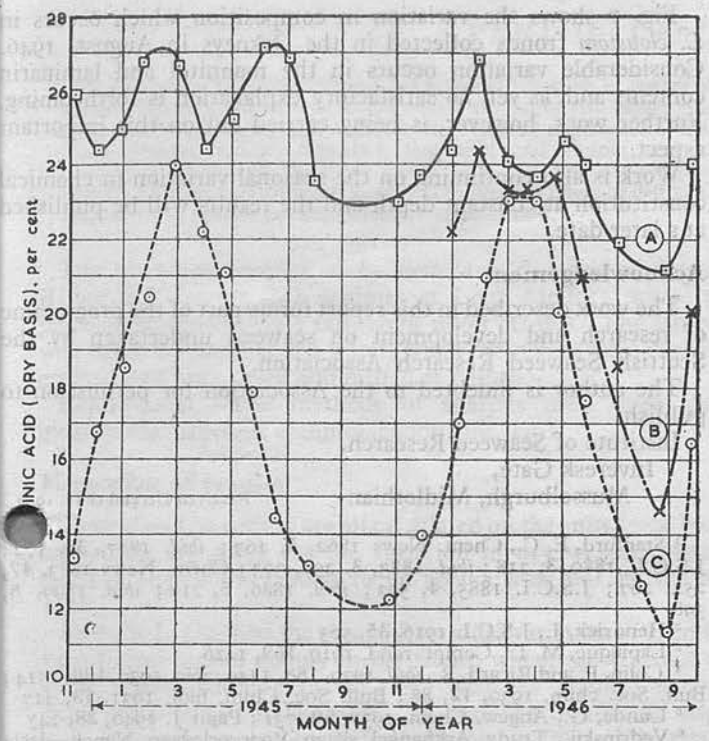

FIG. 5. *Seasonal variation in alginic acid*
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

stuff for the winter. As growth proceeds the wet weight of the frond increases from about 12 oz. in May to 56 oz. in September with an accompanying increase in the dry matter content (Fig. 6), while the weight of the stipe remains practically constant (42 oz.).

Total ash.—Until about 1850 the ash of seaweeds, chiefly of the fucus types, was the sole source of alkali for the soap and glass industry. This kelp industry was then threatened by the Leblanc process, but as the use of iodine in medicine developed, a new kelp industry arose based on the ash of the laminariales, but this industry is also obsolete.

The total ash content of the fronds is at a maximum in May when the old frond is usually cast, 41·3% in 1945 and 43·0% in 1946, and at a minimum in November, 13% in 1944 and 15% in 1945 (Fig. 1). In the stipes the ash varies between 35 and 46% exhibiting maxima in May when the ash is also at a maximum in the frond.

On determining the water-soluble and water-insoluble ash contents of the fronds it was found that the variation occurred in the water-soluble constituents.

Table I

Ash contents of fronds

Sample	% Ash (dry basis) Total ash	Water insoluble	Water soluble	Ash (parts/1000 parts wet weed) Water insoluble	Water soluble
Nov. 1944 ..	12·6	3·51	9·09	6·85	17·74
May 1945 ..	41·3	4·93	.36·35	7·28	53·70

This is in agreement with the results of Lapicque (loc. cit.) who described it as a question of isotonic substitution, the cell sap as it enriched itself in carbohydrates becoming poorer in salts.

Mannitol.—Mannitol, common to all brown seaweeds, was first detected in *L. saccharina* by Stenhouse.[11] It appears to be the primary product of photosynthesis, previous workers having found only traces, or the complete absence of free reducing sugars. It accumulates in the frond during the summer, reaching a maximum of 22% in October, 1945, and 27% in July, 1946. Only slight variations occur in the stipes, minima occurring in the spring when the new frond is forming and when there is little or no photosynthesis (Fig. 4).

Initial experiments have shown that if the plant is kept alive in the dark, and inorganic nitrates are present, proteins are synthesized from mannitol. It is important, therefore, that the plant be dried as soon as collected to prevent or minimize this chemical change.

Laminarin.—A summary of the work which has been carried out on this compound is given by Barry.[12]

It has been shown to be a glucose polymer, composed of a chain of β-glucopyranose units bent into a spiral form.[12] It differs fundamentally, therefore, from starch and cellulose in that the glucose residues are combined by 1 : 3-glycosidic linkages and not through carbon atoms 1 and 4.

The results of this investigation show that all of the laminarin occurs in the frond and does not appear until the mannitol reaches a definite concentration. It is quite probable that it is a secondary product of photosynthesis being formed from mannitol, but it is not known at this stage whether it is a "dark" or a "light" synthesis. It may be formed under the same conditions as starch in the land plants.

It is not known what part it plays in the plant metabolism. Previous investigators have recorded it as a storage foodstuff for the winter and this assumption is supported by the fact that the 13 samples of the annual *saccorhiza bulbosa* which were examined (to be reported later), contained little or no laminarin. It may be, however, that it is utilized in growth. It is at a maximum in the frond in November (28·6% in 1945, Fig. 2), when the total ash and alginic acid are at a minimum.

Alginic acid.—Alginic acid found in all brown seaweeds where it is believed to play an important part in the cell wall was first isolated by Stanford in 1883,[1] and has since been studied by Hoagland and Lieb,[13] Nelson and Cretcher,[14] Gomez,[15] Hirst, Jones, and Jones,[16] Speakman and Chamberlain[17] and Astbury,[18] who found it to be a polyuronide, composed entirely of D-mannuronic acid.

Fig. 5 shows it to be at a maximum in the frond in the spring, 24% in March, 1945, and 23% in March, 1946, and at a minimum

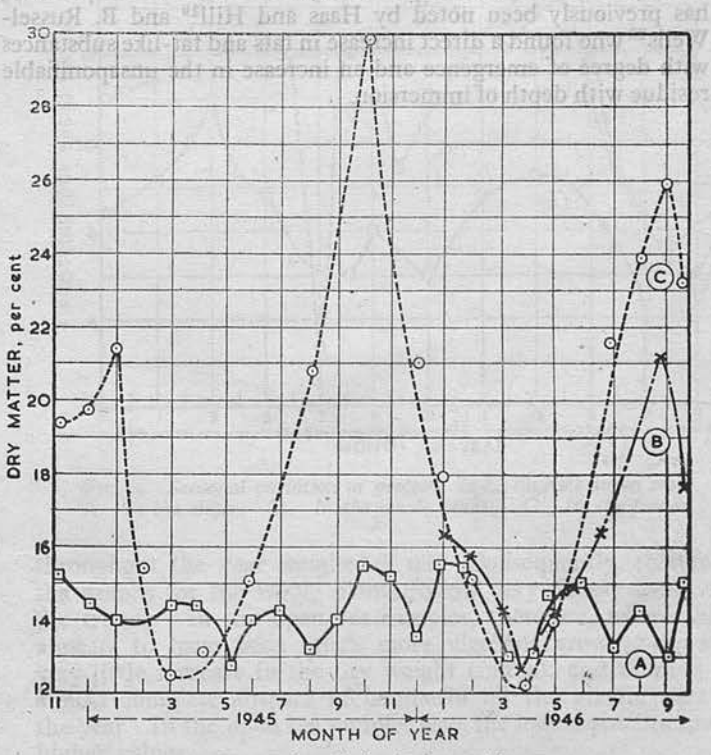

FIG. 6. *Seasonal variation in dry weight content*
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

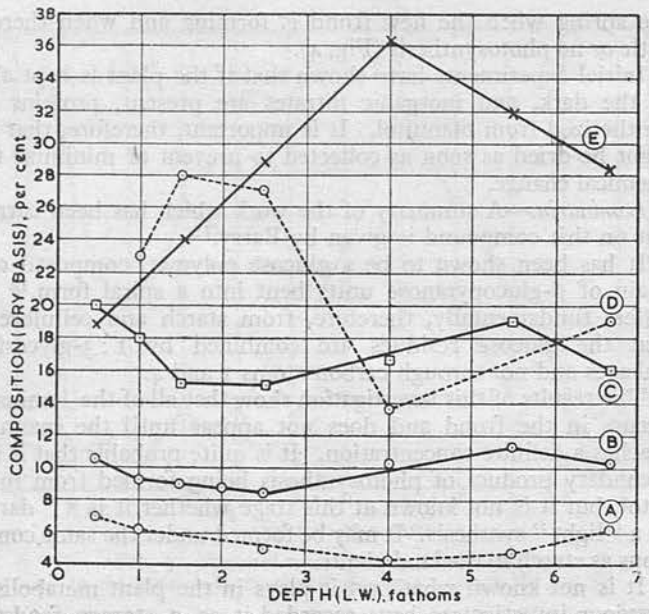

FIG. 7. *Variation in composition of* L. cloustoni *fronds with depth*
A. *Proteins.* B. *Alginic acid.* C. *Total ash.* D. *Laminarin.* E. *Mannitol*

11–12% when the laminarin is at a maximum. In the stipe the alginic acid shows very little seasonal variation.

Proteins.—Very little work has been done on the proteins in seaweed. Fig. 3 shows that in the frond they are at a maximum at the beginning of the year while in the stipe they show very little seasonal variation.

Iodine.—The iodine does undergo some seasonal variation, but it is somewhat erratic. It varies between 0·5 and 1·0%, being at a maximum in the spring, and in general it is higher in the frond than in the stipe.

Variation in composition with depth of immersion.

The relationship between the depth of immersion of algae and their metabolism as expressed by their chemical composition, has previously been noted by Haas and Hill[19] and B. Russel-Wells[20] who found a direct increase in fats and fat-like substances with degree of emergence and an increase in the unsaponifiable residue with depth of immersion.

Fig. 7 shows the variation in composition which occurs i *L. cloustoni* fronds collected in the Orkneys in August, 194 Considerable variation occurs in the mannitol and laminar contents and, as yet, no satisfactory explanation is forthcomin Further work, however, is being carried out on this import aspect.

Work is also continuing on the seasonal variation in chemic constitution at constant depth and the results will be publish at a later date.

Acknowledgement

The work described in this report forms part of the programm of research and development on seaweed undertaken by t Scottish Seaweed Research Association.

The author is indebted to the Association for permission publish.

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Received December 15, 19

[1] Stanford, E. C., Chem. News 1862, **5**, 167; *ibid.* 1877, **35**, 1 J.S.C.I., 1880, **3**, 218; *ibid.* 1883, **3**, 297, 303; Chem. News 1883, 4 254, 267; J.S.C.I. 1885, **4**, 592; *ibid.* 1886, **5**, 218; *ibid.* 1899, 398
[2] Hendrick, J., J.S.C.I. 1916, **35**, 565
[3] Lapicque, M. L., Compt. rend. 1919, **169**, 1426
[4] Colin, P. and Ricard, R., *ibid.* 1929, **188**, 1449; *ibid.* 1930, **190**, 151 Bull. Soc. chim. 1930, **12**, 88; Bull. Soc. Chim. biol. 1931, **13**, 41
[5] Lunde, G., Angew. Chem. 1937, **50**, 731; Papir J. 1940, **28**, 147
[6] Vedrinskii, Trudy Arkhangel skago Vodoroslevago Nanch—Is dovatel Inst. Vodorosli Belago Morya. 1938, **61**, 79
[7] Atsuki and Tomoda, J. Soc. Chem. Ind. Japan 1926, **29**, 509, 599
[8] Dillon, T., Chem. Age 1943, **49**, 279
[9] Baggesguard-Rasmusses, Chem. Zentr. 1941, **11**, 2894
[10] Cameron, M. C., Ross, A. G., and Percival, E. G. V., J.S.C.I. 194 **67**, 161
[11] Stenhouse, J., Ann. Chim. Farm. 1844, **51**, 349
[12] Barry, V., Sci. Proc. Roy. Dublin, Soc. 1938, **21**, 615
[13] Hoagland and Lieb, J. Biol. Chem. 1915, **23**, 287
[14] Nelson and Cretcher, J. Amer. Chem. Soc. 1929, **51**, 1914
[15] Gomez, M. L., Inst. Espan. Oceanograf. Notas y Resumenes S II, 1933, **74**, 98
[16] Hirst, Jones, and Jones (Miss), J.C.S. 1939, 1880
[17] Speakman, J. B. and Chamberlain, N. H., J. Soc. Dyers and C 1944, **60**, 265
[18] Astbury, W. T., Nature 1945, 155
[19] Haas, P. and Hill, T. G., Ann. Bot. 1933, **47**, 55
[20] Russel-Wells, B., Nature 1932, **129**, 654

PART II. *LAMINARIA DIGITATA*

In a previous communication,[1] the seasonal variation in chemical constitution of *Laminaria cloustoni* over a period of two years was given. The present paper outlines the seasonal variation in ash, crude protein, iodine, mannitol, laminarin and alginic acid of *Laminaria digitata* for the same period. In this case the samples were taken from two different localities, to find the effect of different degrees of exposure on the chemical constitution.

The open sea samples of *L. digitata* were taken at Atlantic Bridge and the sea loch samples at Loch Melfort, where the plants were growing in ½ fathom of water (L.W.). The samples were taken by hand at low water, when the plants were partially exposed and were dried and milled as before.[1]

Experimental.—The methods of analysis used were those given in the previous communication (loc. cit.).

Discussion of results

General.—The results are all calculated on the anhydrous basis, but graphs are given for the variation in dry weight content, enabling any of the results to be recalculated out on the wet weed.

As with *L. cloustoni* the marked seasonal variation in chemical constitution occurs mainly in the frond, where most, if not all, of the photosynthesis occurs, but with *L. digitata* maxima occur much earlier in the year. In 1946 the fronds and stipes were weighed separately, before drying, enabling the results to be calculated out on the whole plant. During the summer a considerable increase in the weight of the wet frond occurs, particularly in the open sea samples, while very little change occurs in the weight of the stipes. Thus, in the open sea, fronds weighing 76–80 oz. were obtained, while the loch samples weighed only 30–40 oz. In both cases, however, the stipes throughout the year weighed 8 oz. Consequently, therefore, the graphs for the whole plants follow very closely those for the fronds. In the open sea samples, therefore, where there appears to have been much more vigorous growth, there is very little increase in the dry weight content, and there is an almost complete absence of laminarin for the greater part of the year. In the open sea samples also, the iodine reaches much higher values.

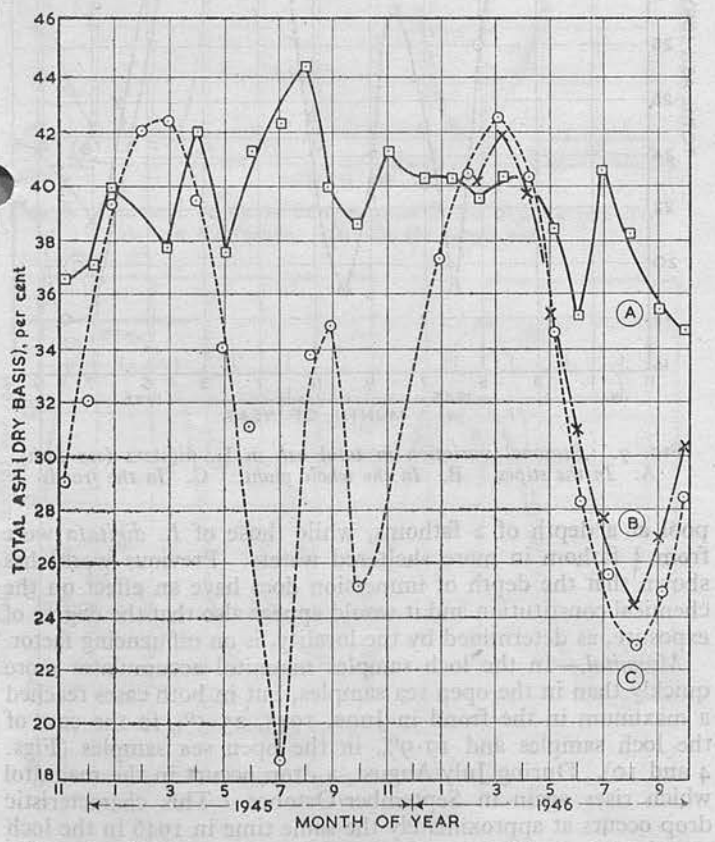

FIG. 1. *Seasonal variation in total ash in* L. digitata (*open sea*). A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

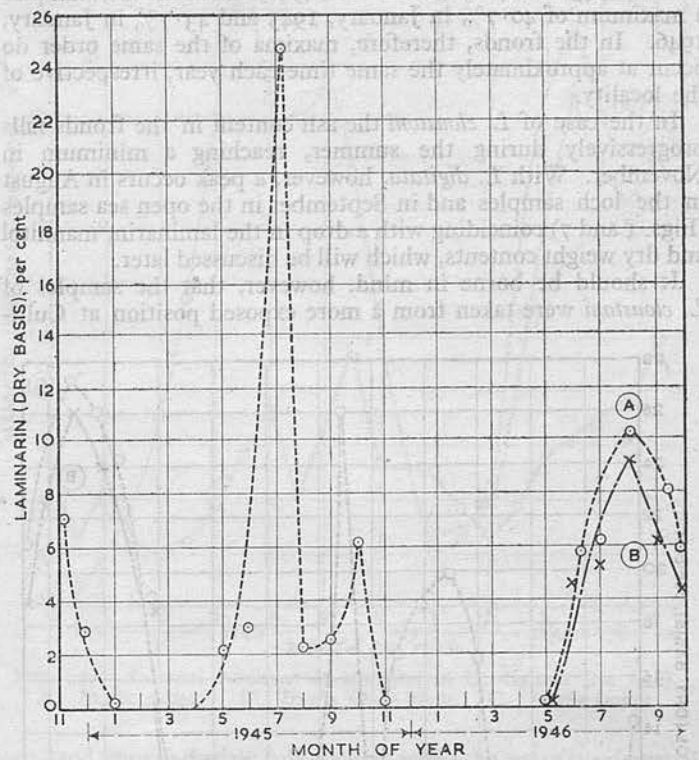

FIG. 2. *Seasonal variation in laminarin in* L. digitata (*open sea*). A. *In the fronds.* B. *In the whole plant*

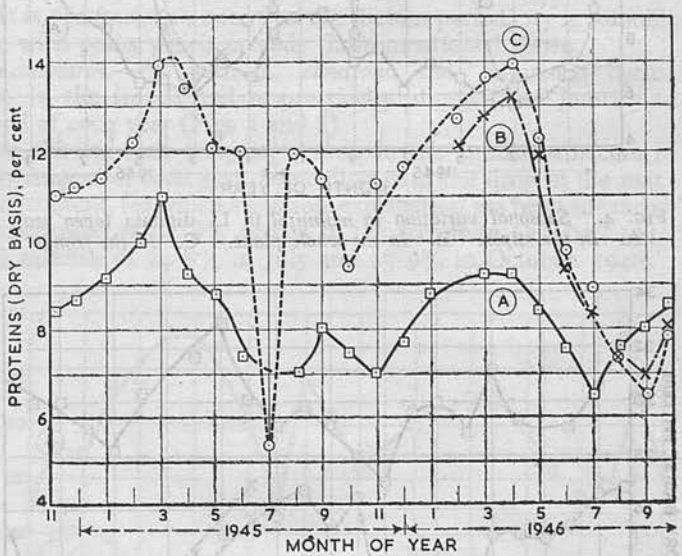

FIG. 3. *Seasonal variation in proteins in* L. digitata (*open sea*). A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

Total ash.—In both the open sea and loch samples the ash when calculated on the anhydrous basis is at a maximum in the

frond from January to March, before photosynthesis commences. In the open sea samples it reaches a maximum of 42·4% in March, 1945, and 42·5% in March, 1946, and in the loch samples a maximum of 40·7% in January, 1945 and 43·7% in January, 1946. In the fronds, therefore, maxima of the same order do occur at approximately the same time each year, irrespective of the locality.

In the case of *L. cloustoni* the ash content in the fronds falls progressively during the summer, reaching a minimum in November. With *L. digitata*, however, a peak occurs in August in the loch samples and in September in the open sea samples (Figs. 1 and 7) coinciding with a drop in the laminarin, mannitol and dry weight contents, which will be discussed later.

It should be borne in mind, however, that the samples of *L. cloustoni* were taken from a more exposed position at Culli-

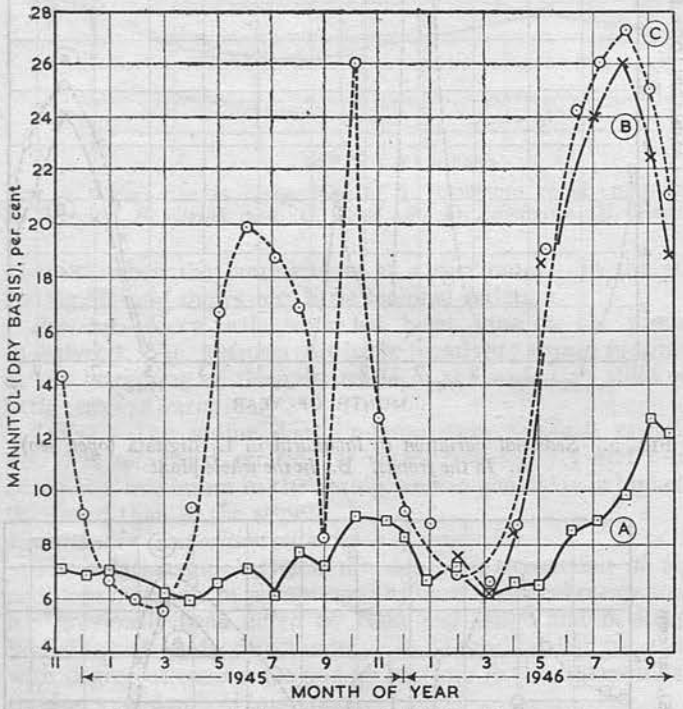

FIG. 4. *Seasonal variation in mannitol in* L. digitata *(open sea).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

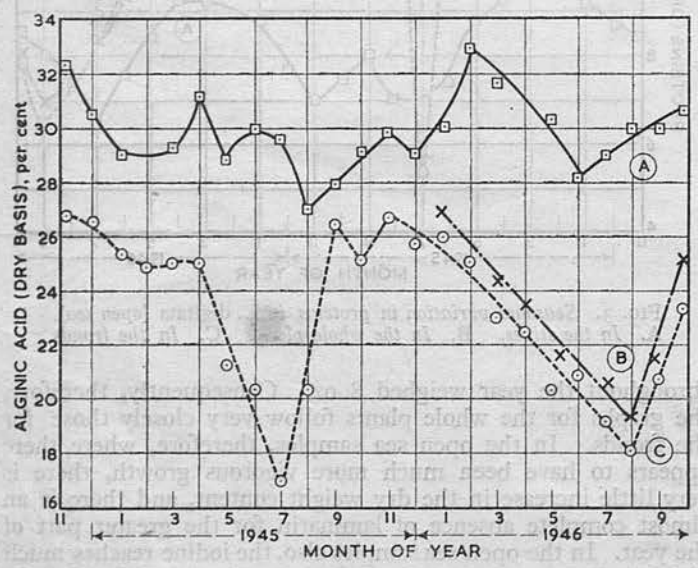

FIG. 5. *Seasonal variation in alginic acid in* L. digitata *(open sea).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

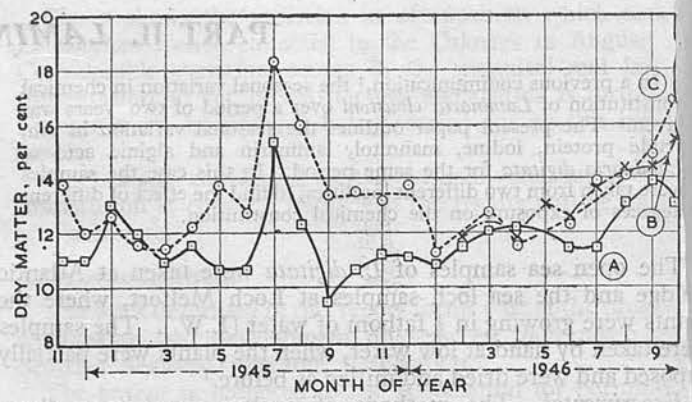

FIG. 6. *Seasonal variation in dry matter content in* L. digitata *(open sea).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

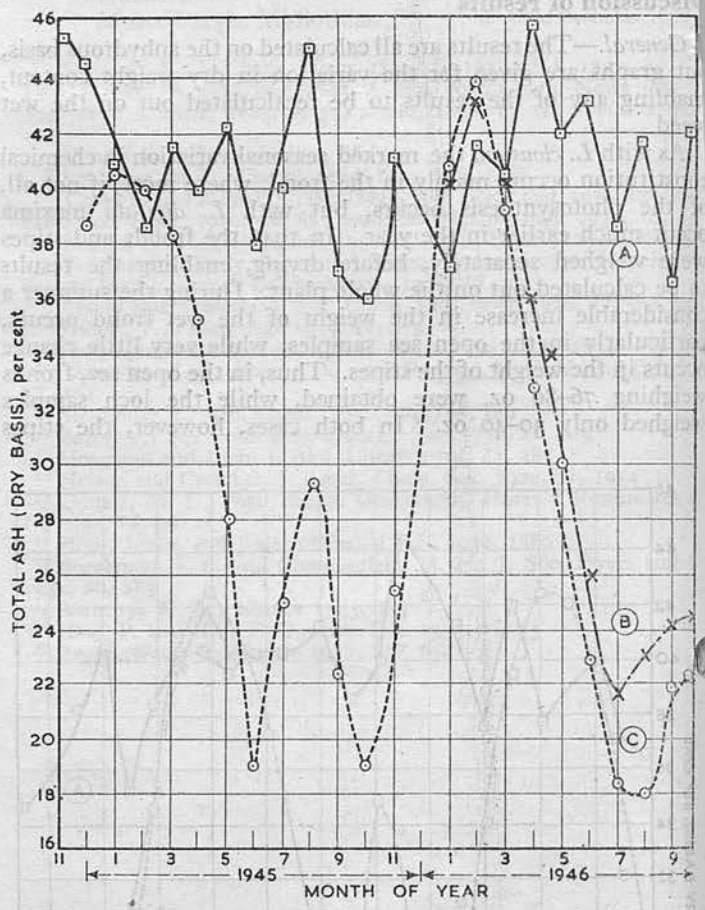

FIG. 7. *Seasonal variation in total ash in* L. digitata *(sea loch).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

pool at a depth of 2 fathoms, while those of *L. digitata* were from ½ fathom in more sheltered waters. Previous work[1] has shown that the depth of immersion does have an effect on the chemical constitution and it would appear also that the degree of exposure, as determined by the locality, is an influencing factor.

Mannitol.—In the loch samples mannitol accumulates more quickly than in the open sea samples, but in both cases reached a maximum in the frond in June, 1945, 25·5% in the case of the loch samples and 19·9% in the open sea samples (Figs. 4 and 10). During July/August, a drop occurs in the mannitol which rises again in September/October. This characteristic drop occurs at approximately the same time in 1946 in the loch samples but not in the open sea samples. A possible explanation

is that this falling off in photosynthesis may be due to the absence of nutrients such as nitrates and phosphates in the water. It is well known that the growth rate of plants in the sea is reduced by low concentrations of nutrient salts in solution in the water, and it is the rate of replenishments of the nutrients in the water of the photosynthetic zone which ultimately limits the production. Experiments with diatoms,[2] have shown a marked reduction in the rate of photosynthesis with phosphate concentrations below about 10 mg. phosphorus per cubic metre. It is possible, therefore, that there are three periods of growth, a period of rapid growth during the spring and early summer when the nitrate and phosphate content of the water is high, a period of restricted growth during July/August when the water is exhausted of nitrates and phosphates and a third period in September/October when the nitrates and phosphates are again regenerated in the photosynthetic layer.

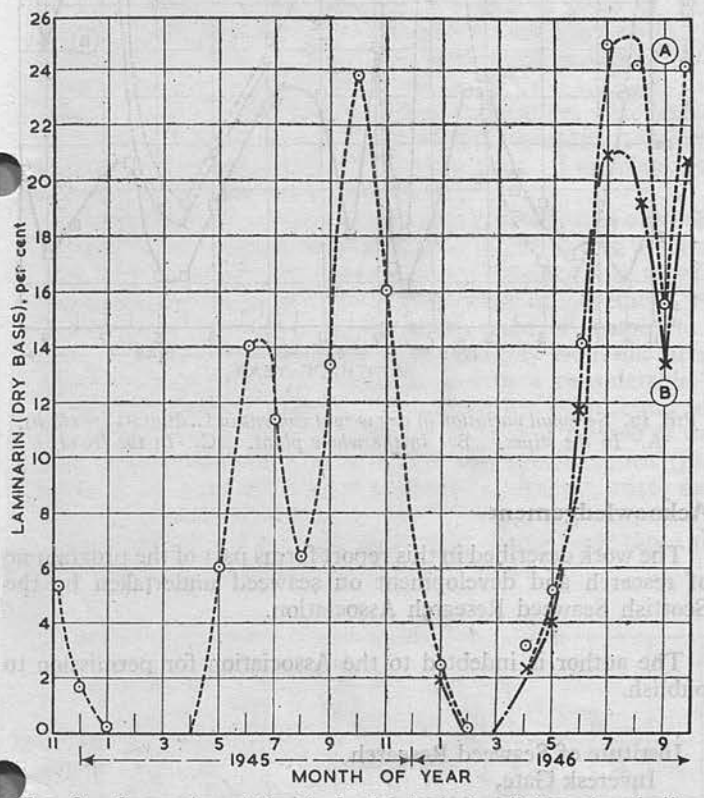

FIG. 8. *Seasonal variation in laminarin in* L. digitata *(sea loch).* A. *In the fronds.* B. *In the whole plant*

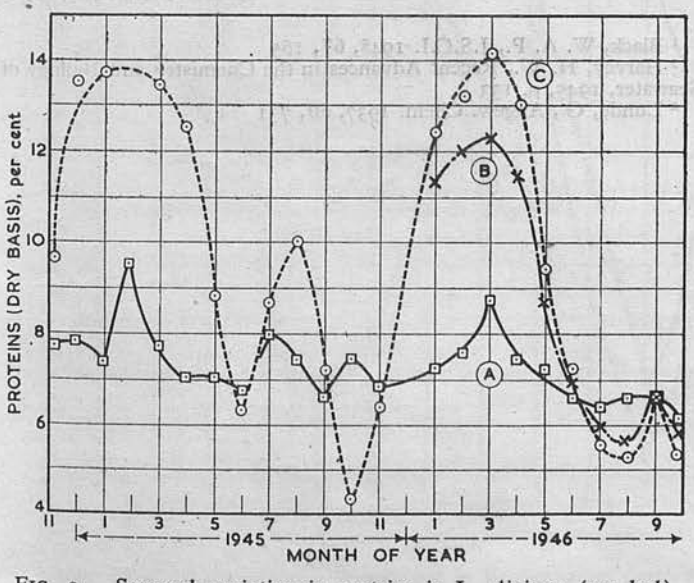

FIG. 9. *Seasonal variation in proteins in* L. digitata *(sea loch).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

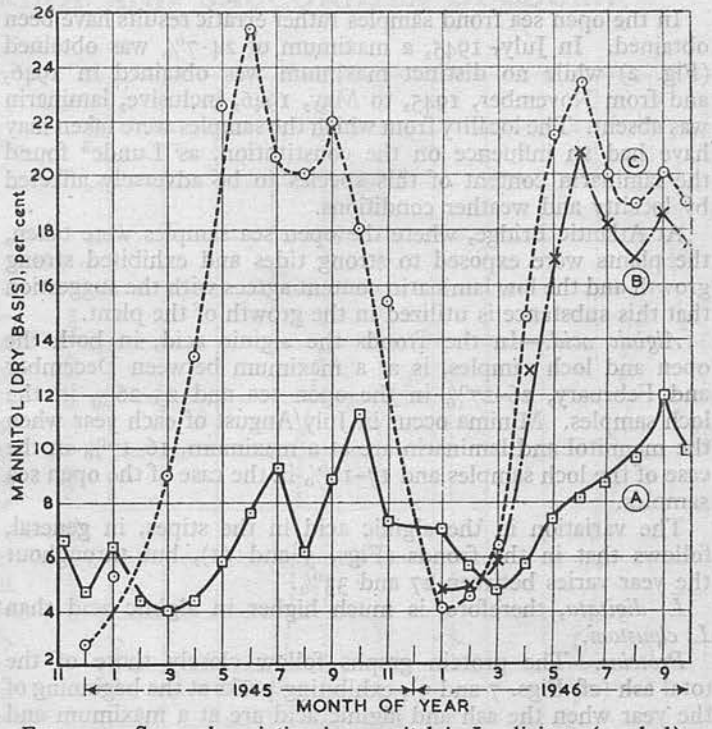

FIG. 10. *Seasonal variation in mannitol in* L. digitata *(sea loch).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

It is of interest to note that the diatom periodicity is a double one with peaks which precede those mentioned above.

Laminarin.—As with *L. cloustoni*, the laminarin occurs only in the fronds and is practically absent from January to April of each year (Figs 2 and 8).

In the sea loch samples, a drop in the laminarin occurs in July/August of each year, coinciding with the drop in the mannitol content, for which a possible explanation has been advanced. A maximum of 23·8% occurs in the fronds in October, 1945, and maxima of 24·8% in July and 23·9% in October, 1946.

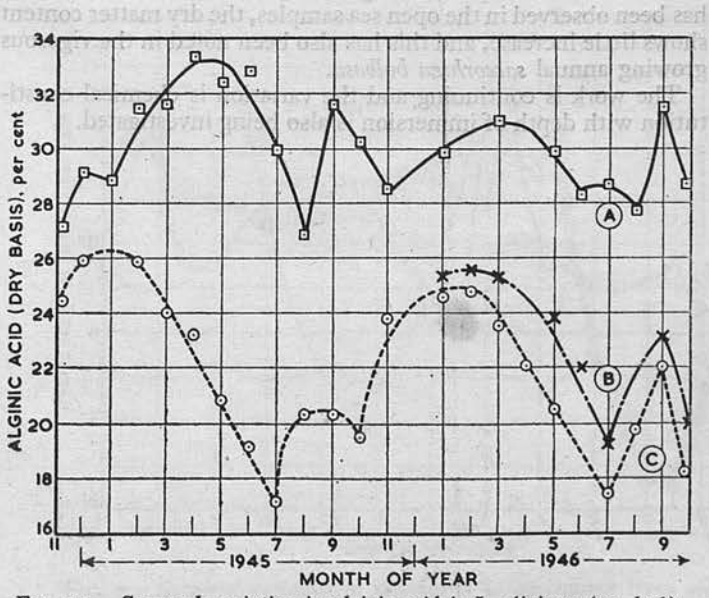

FIG. 11. *Seasonal variation in alginic acid in* L. digitata *(sea loch).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

In the open sea frond samples rather erratic results have been obtained. In July, 1945, a maximum of 24·7% was obtained (Fig. 2) while no distinct maximum was obtained in 1946, and from November, 1945, to May, 1946, inclusive, laminarin was absent. The locality from which the samples were taken may have had an influence on the constitution, as Lunde[3] found the laminarin content of this species to be adversely affected by locality and weather conditions.

At Atlantic Bridge, where the open sea samples were taken, the plants were exposed to strong tides and exhibited strong growth and the low laminarin content agrees with the suggestion that this substance is utilized in the growth of the plant.

Alginic acid.—In the fronds the alginic acid, in both the open and loch samples, is at a maximum between December and February, 26–27% in the open sea and 25–26% in the loch samples. Minima occur in July/August of each year when the mannitol and laminarin are at a maximum, 16–17% in the case of the loch samples and 17–18% in the case of the open sea samples.

The variation in the alginic acid in the stipes, in general, follows that in the fronds (Figs. 5 and 11), but throughout the year varies between 27 and 33%.

L. digitata, therefore, is much higher in alginic acid than *L. cloustoni*.

Proteins.—The protein graphs follow closely those of the total ash (cf. Figs. 7 and 9), exhibiting peaks at the beginning of the year when the ash and alginic acid are at a maximum and the laminarin and mannitol are at a minimum. At the end of the year when the mannitol content begins to fall, the protein content increases. Proteins are a product of "dark" synthesis and previous work[1] has shown that they are probably synthesized from mannitol.

Iodine.—The iodine undergoes seasonal variation in the fronds, being at a maximum at the beginning of the year when the ash is at a maximum, 0·8–1·1% in the open sea samples and 0·6–0·7% in the loch samples. In the stipes the variation is between 0·3 and 0·7%.

Dry matter content.—As with *L. cloustoni*[1] the dry matter graphs for the fronds follow the laminarin graph (Figs. 2, 6, 8 and 12), there being no increase in the dry matter content until laminarin is formed. In the open sea samples, (Fig. 6) there is not the same seasonal variation as in the loch samples (Fig. 12), on account of the absence of, or low, laminarin content.

Where there is rapid and vigorous growth, however, and this has been observed in the open sea samples, the dry matter content shows little increase, and this has also been noted in the vigorous growing annual *saccorhiza bulbosa*.

The work is continuing and the variation is chemical constitution with depth of immersion is also being investigated.

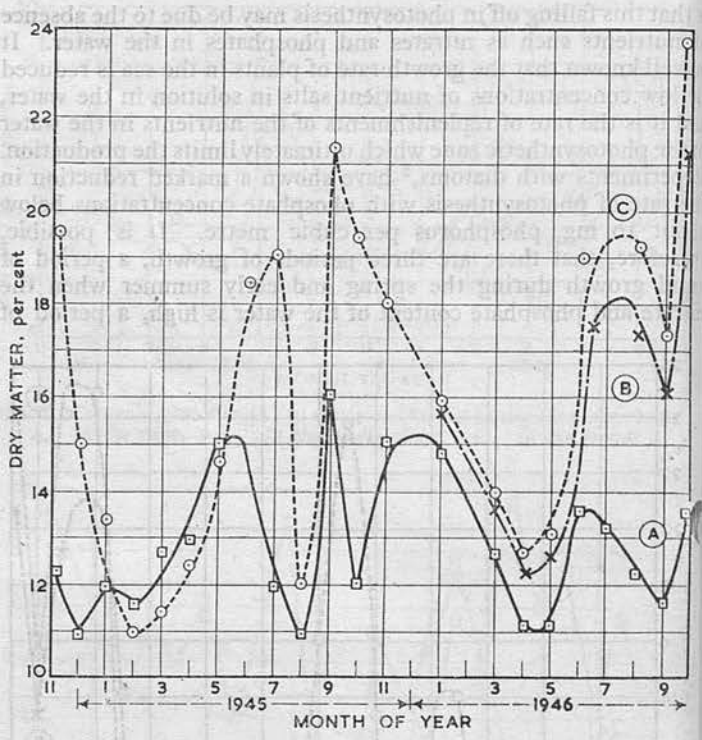

FIG. 12. *Seasonal variation in dry weight content in* L. digitata *(sea loch).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

Acknowledgement

The work described in this report forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association.

The author is indebted to the Association for permission to publish.

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Received December 15, 1947

[1] Black, W. A. P., J.S.C.I. 1948, **67**, 165
[2] Harvey, H. W., Recent Advances in the Chemistry and Biology of Seawater, 1945, p. 133
[3] Lunde, G., Angew. Chem. 1937, **50**, 731

PART III. *LAMINARIA SACCHARINA* AND *SACCORHIZA BULBOSA*

In previous communications,[1, 2] the seasonal variation in chemical constitution of the laminariales *cloustoni* and *digitata* over a period of two years was recorded. The present paper outlines the seasonal variation in ash, crude protein, iodine, mannitol, laminarin and alginic acid of *L. saccharina* for the same period. As with *L. digitata*, the samples were taken from two different localities, to find the effect of different degrees of exposure on the chemical constitution, and the stipes and fronds have been analysed separately. Samples of the annual *saccorhiza bulbosa* have also been analysed.

The open sea samples of *L. saccharina* were taken at Rudh-an-Aoil, Shuna Island, and the sea loch samples at Eilean Coltair, Loch Melfort where the plants were growing in 1½ to 2 fathoms of water (L.W.). The samples of *saccorhiza bulbosa* were taken at a depth of 2 fathoms (L.W.) at Cullipool, Luing Island. As in the case of *L. cloustoni*, the samples were obtained by trawling a multi-pronged grapnel for 2 minutes, hoisting the grapnel and lifting the weed into the boat. Two plants were selected, dried and analysed as before.[1]

Saccorhiza bulbosa is an annual which generally dies away about November, although it has been known to persist into the second year.[3] In November, 1945, a sample was obtained with very little frond, and the wings practically gone, while in November, 1946, it was found impossible to obtain any kind of sample, as the plant appeared to have disappeared entirely from the area in which it had grown in profusion and to a considerable size during the summer months. This weed has been found to be one of the first to grow to any size in an area cleared of weed. The rapidity and the size to which this species grows (plants up to 48 lb. were obtained at Cullipool in August, 1946) makes this alga of especial interest.

Experimental.—The methods of analysis used were those given in a previous communication.[1]

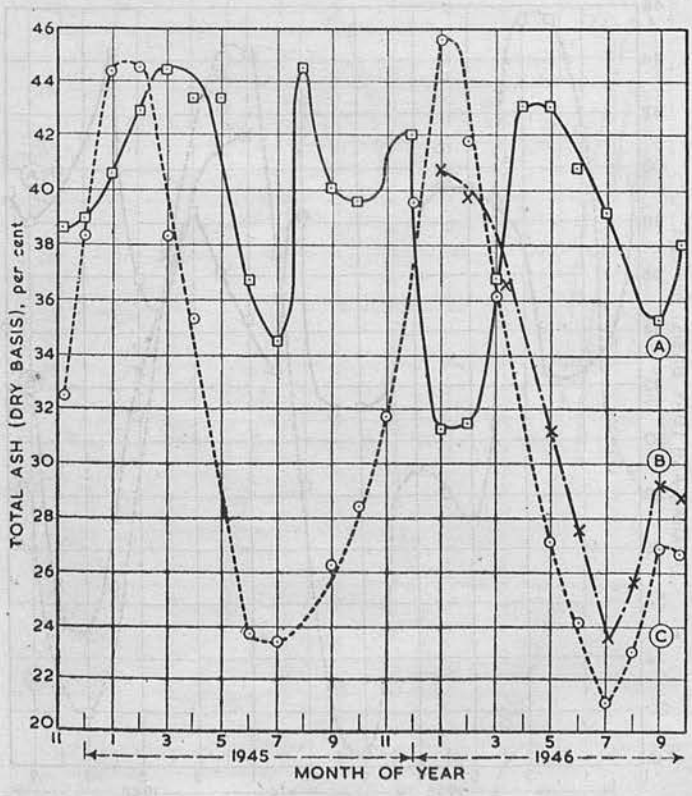

FIG. 1. *Seasonal variation in total ash in* L. saccharina *(open sea).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

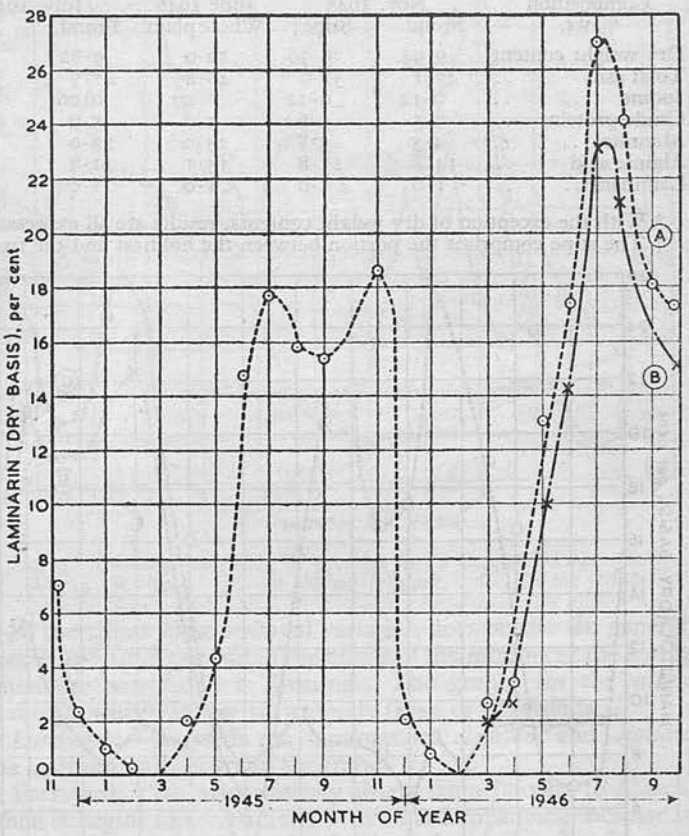

FIG. 2. *Seasonal variation in laminarin in* L. saccharina *(open sea).* A. *In the fronds.* B. *In the whole plant*

Discussion of results

General.—The results are all expressed on the anhydrous basis and in 1946 from the weights of the stipes and fronds before analysis, the results have also been calculated out on the whole plant. Graphs are also given for the variation in the dry weight content so that any of the results can be recalculated out on the wet weed.

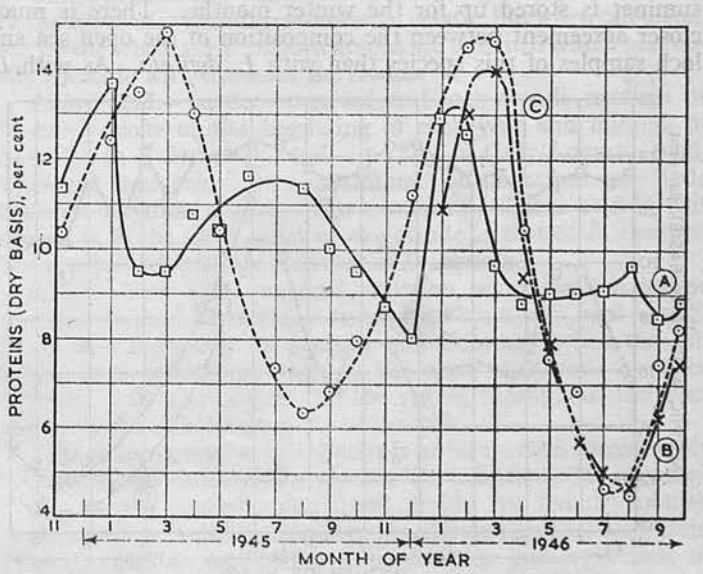

FIG. 3. *Seasonal variation in proteins in* L. saccharina *(open sea).* A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

Table I

Seasonal variation in composition of saccorhiza bulbosa*

Composition % wt.	Nov. 1945 Frond	Nov. 1945 Stipe†	June 1946 Whole plant	July 1946 Frond	July 1946 Stipe	Aug. 1946 Frond	Aug. 1946 Stipe	Sept. 1946 Frond	Sept. 1946 Stipe	Oct. 1946 Frond	Oct. 1946 Stipe
Dry weight content	9·94	9·76	10·0	9·82	7·39	11·03	9·68	10·27	8·89	—	—
Total ash ..	47·1	51·0	48·8	41·7	55·0	32·1	42·3	38·9	31·5	37·6	35·1
Iodine	0·12	0·12	0·07	0·06	0·09	0·10	0·10	0·05	0·13	0·16	0·11
Crude proteins ..	7·5	5·6	5·0	5·9	3·5	5·9	4·8	4·9	5·4	6·2	5·6
Mannitol	4·5	5·8	15·0	22·0	11·9	28·2	23·1	21·7	21·5	16·0	19·5
Alginic acid ..	14·8	15·8	13·1	11·8	14·5	12·9	13·1	14·0	14·6	15·7	15·6
Laminarin.. ..	<1·0	<1·0	<1·0	<1·0	<1·0	<1·0	<1·0	<1·0	<1·0	<1·0	<1·0

* With the exception of dry weight contents, results are all expressed on anhydrous basis
† The stipe comprises the portion between the holdfast and the frond

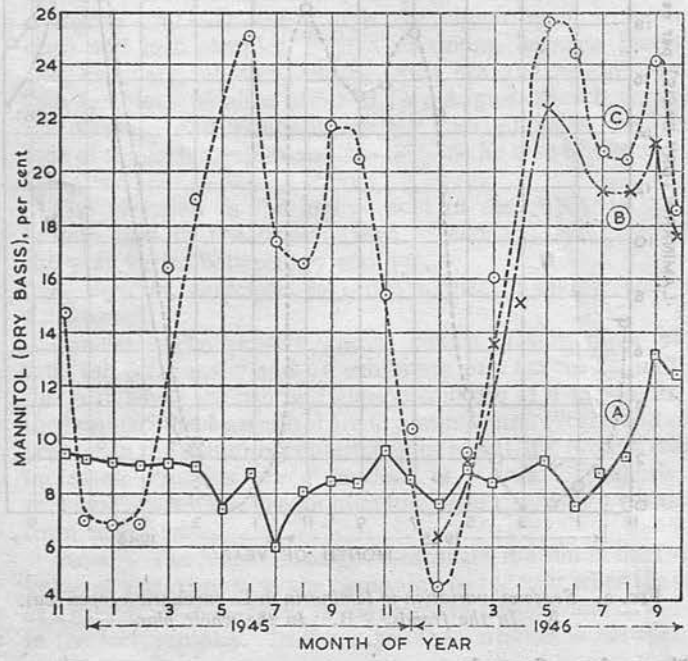

FIG. 4. *Seasonal variation in mannitol in* L. saccharina *(open sea).*
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

In *L. saccharina* as in the laminariales *cloustoni* and *digitata* (*loc. cit.*) the marked seasonal variations in composition which have been previously recorded[4] are due almost entirely to changes in the composition of the fronds where photosynthesis occurs and where the bulk of the carbohydrates synthesized during the summer is stored up for the winter months. There is much closer agreement between the composition of the open sea and loch samples of this species than with *L. digitata.* As with *L.*

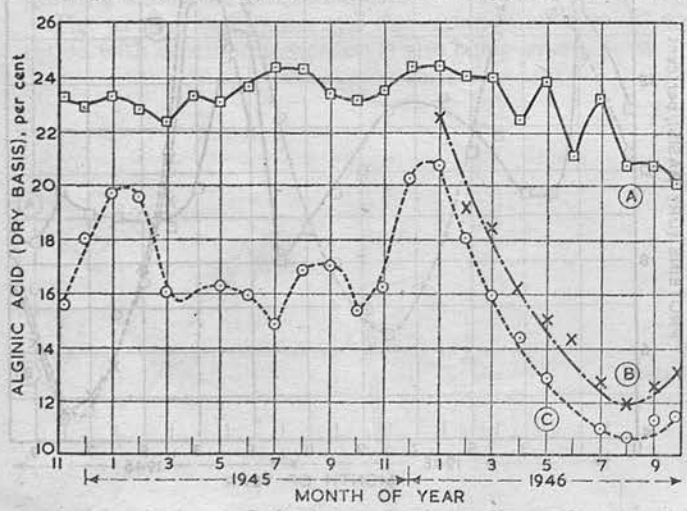

FIG. 5. *Seasonal variation in alginic acid in* L. saccharina *(open sea).*
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

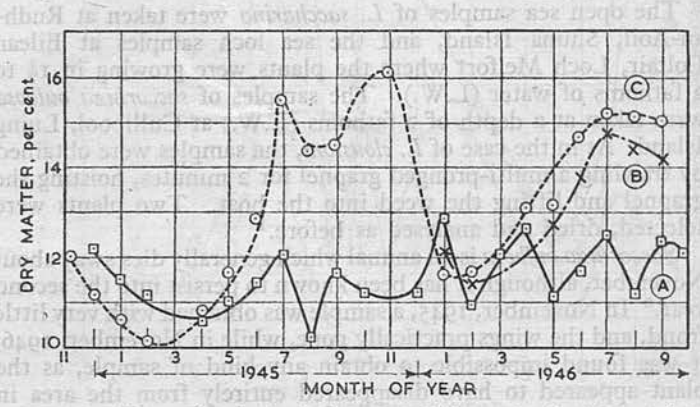

FIG. 6. *Seasonal variation in dry matter in* L. saccharina *(open sea).*
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

digitata, however, much larger plants were obtained from the open sea position. The samples of *L. saccharina* were all taken from depths of 1½–2 fathoms and were not exposed at low water as was the case with *L. digitata.*

It has been shown with *L. cloustoni* (loc. cit.) that variations in composition do occur with depth of immersion, and this may

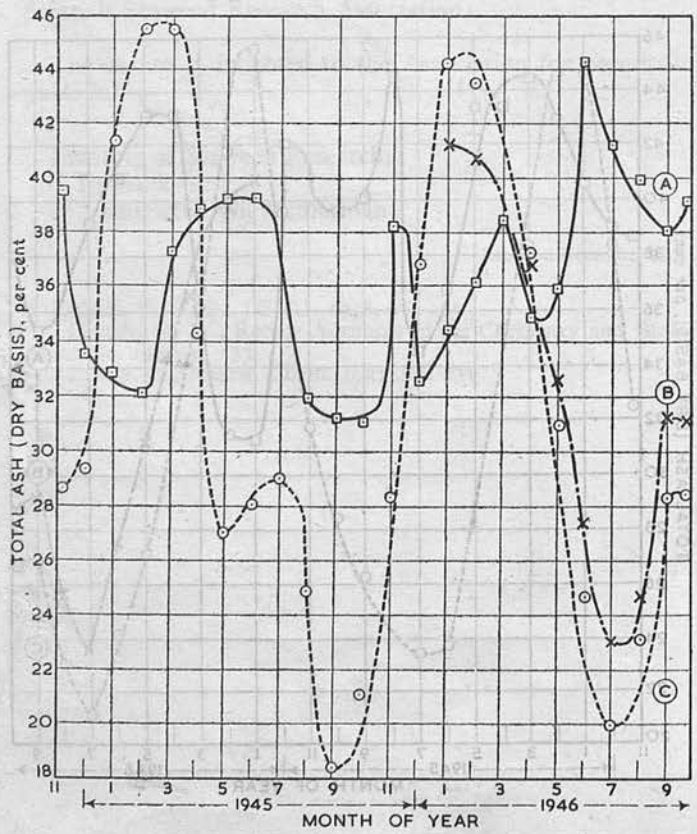

FIG. 7. *Seasonal variation in total ash in* L. saccharina *(sea loch).*
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

account for slight difference in composition between these three species coupled with the fact that the *L. digitata* is exposed at low water and therefore undergoes desiccation which no doubt has an influence on photosynthesis.

Total ash.—The open sea fronds have maximum ash content of 44–45% from January/February, 1945, and 45·7% in January, 1946, and minimum ash contents of 22–23% in June/July, 1945, and 21–24% in June/August, 1946, while in the loch fronds maxima of 45–46% occur in February/March, 1945, and 43–44% in January/February, 1946, and minima of 18·2% in September, 1945, and 20% in July, 1946. Maxima and minima of the same order, therefore, occurs at approximately the same time of year as in *L. digitata*, but at an earlier period than in *L. cloustoni*.

The variation in the stipes is somewhat erratic but when the total ash was calculated on the whole plant a smooth graph was obtained (Figs. 1 and 7), maxima occurring in January, 40·5% for the open sea sample and 41·0% for the sea loch sample, and minima in July, 23·5% for the open sea and 22·5% for the loch samples.

On determining the water soluble and water insoluble ash contents of the fronds it was found as in the case of *L. cloustoni* that the seasonal variation in total ash occurred mainly in the water soluble constituents.

Mannitol.—In the fronds minima occur from January/February of each year, before photosynthesis commences and when the mannitol synthesized during the previous summer has practically all been utilized (Figs. 4 and 10). The mannitol then accumulates in the fronds and reaches a maximum of 24–26% in May/June of each year. In July/August of each year, a drop occurs in the mannitol and an explanation has been advanced in a previous paper.[2] As with *L. digitata*, an increase in the mannitol occurs again in September, which no doubt coincides with regeneration of nutrient salts such as nitrates and phosphates in the photosynthetic layer.

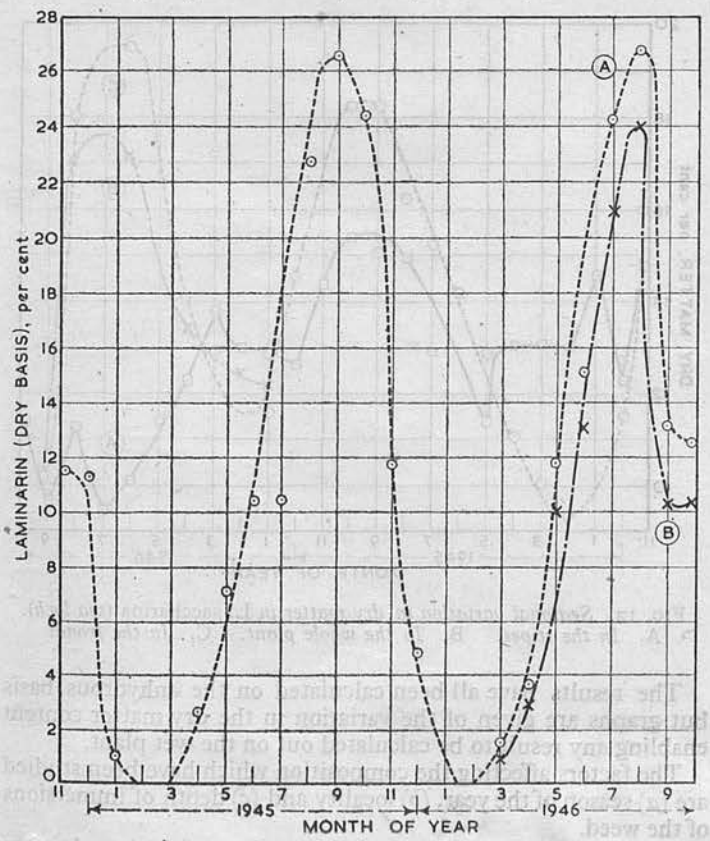

FIG. 8. *Seasonal variation in laminarin in* L. saccharina (*sea loch*).
A. *In the fronds.* B. *In the whole plant*

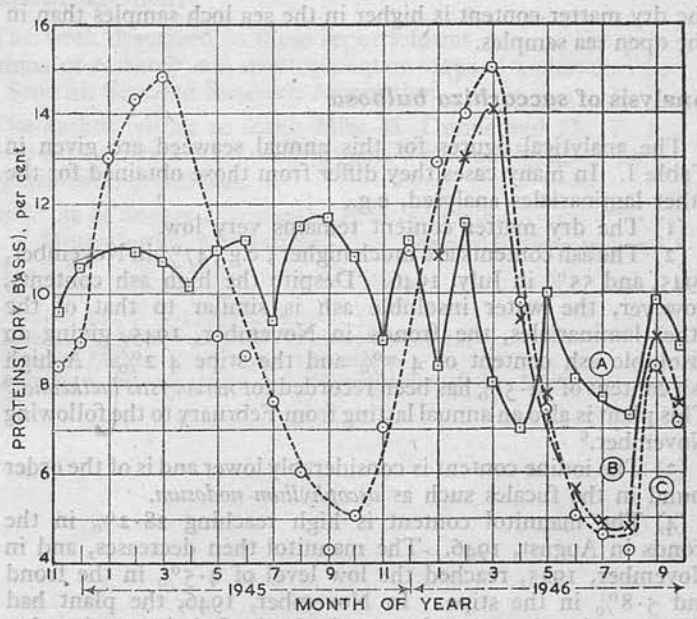

FIG. 9. *Seasonal variation in proteins in* L. saccharina (*sea loch*).
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

In the stipes some seasonal variation does occur. In general, maxima occur at the end of the year and the minima in the spring when the new frond is forming. The graphs for the whole plants, however, follow very closely those of the fronds.

Laminarin.—As with the laminariales *cloustoni* and *digitata* the laminarin all occurs in the frond.

Each year, it is almost entirely absent from January to March, when it begins to accumulate, with an accompanying increase in the dry matter content. In the loch frond samples, in 1945 the laminarin reaches a maximum of 26·5% in September, and 26·7% in August, 1946, which is equivalent to 24% when calculated on the whole plant (Figs. 2 and 8). In the open sea frond samples, maxima of 15·8% and 18·5% occur in August and November, 1945, and a maximum of 26·9% in July, 1946.

Crude proteins.—The frond protein graphs, (Figs. 3 and 9) for the open sea and loch samples are very similar, exhibiting maxima of 14–15% in March of each year. The graphs also follow those for the total ash (Figs. 1 and 7), and are the inverse of the mannitol and laminarin graphs (Figs. 2, 4, 8 and 10). In the stipes the variation is somewhat erratic, but when calculated out on the whole plant smooth graphs are obtained which practically coincide with those for the fronds.

Alginic acid.—In the open sea and loch fronds maxima of 18–20% occur at the beginning of each year and minima of 14–16% in July, 1945, and 10–12% in July/August, 1946, when the laminarin is at a maximum. In the stipes very little seasonal variation occurs. The percentage alginic acid in this species is of the same order of magnitude as that of *L. cloustoni* but is appreciably lower than that of *L. digitata*.

Iodine.—Very little seasonal variation occurs in the iodine content. In the loch fronds it varies between 0·20% and 0·50% being at a maximum in January and February when the ash is also at a maximum, while in the open sea fronds it varies between 0·25 and 0·50% In the stipes, throughout the year the variation is between 0·20 and 0·50%.

The iodine content of this species is on an average, considerably lower than that of *L. digitata* which is less than that of *L. cloustoni*.

Dry matter content.—The frond graphs for the dry matter contents (Figs. 6 and 12) follow very closely those for laminarin (Figs. 2 and 8)—exhibiting minima from January/March of each year when laminarin is absent and maxima when the laminarin is at a maximum. In general as was the case with *L. digitata*

the dry matter content is higher in the sea loch samples than in the open sea samples.

Analysis of *saccorhiza bulbosa*

The analytical figures for this annual seaweed are given in Table I. In many cases they differ from those obtained for the other laminariales analysed, e.g.,

(1) The dry matter content remains very low.

(2) The ash contents are much higher; e.g., 47% in November, 1945, and 55% in July, 1946. Despite the high ash contents, however, the water insoluble ash is similar to that of the other laminariales, the fronds in November, 1945, giving an insoluble ash content of 4·7% and the stipe 4·2%. A high ash content of 54·5% has been recorded for *nereocystis luetkeana*.[5] This plant is also an annual lasting from February to the following November.[6]

(3) The iodine content is considerably lower and is of the order found in the fucales such as *ascophyllum nodosum*.

(4) The mannitol content is high reaching 28·2% in the fronds in August, 1946. The mannitol then decreases, and in November, 1945, reached the low level of 4·5% in the frond and 5·8% in the stipe. In November, 1946, the plant had disappeared and no sample was obtained. It is interesting that this annual should build up such a large reserve of mannitol. It is a plant which grows very rapidly and to an enormous size in a few months, and a considerable amount of mannitol, etc., will no doubt be required to sustain this growth. Before November, however, the mannitol has practically disappeared, and it may be that mannitol and higher synthetic products have been used up in sporing, and to maintain life in its final stages when photosynthesis had ceased.

(5) The alginic acid content is relatively low 13–16% (on the anhydrous basis) but it is comparable to the amount in *L. saccharina* at that time of the year.

(6) Laminarin is absent.

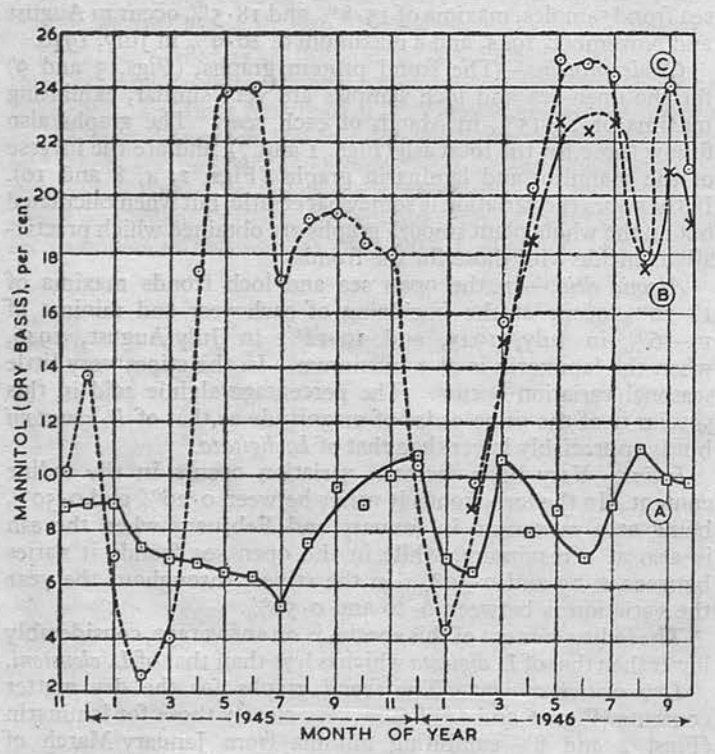

FIG. 10. *Seasonal variation in mannitol in* L. saccharina (*sea loch*).
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

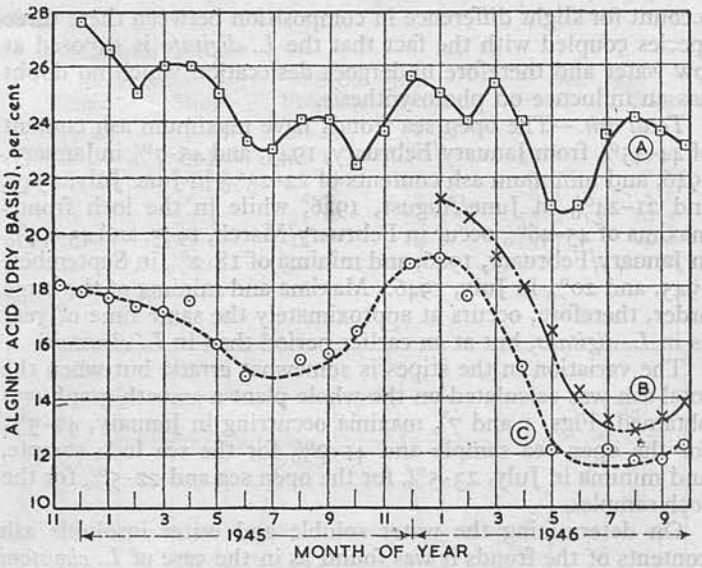

FIG. 11. *Seasonal variation in alginic acid in* L. saccharina (*sea loch*).
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

Summary of seasonal variation in composition of the laminariales *cloustoni, digitata* and *saccharina*

The variations in chemical composition of the laminariales *cloustoni*, *digitata* and *saccharina*, over a period of two years, have been studied. ([1, 2] and present communication.)

Monthly samples of the species have been collected, the plants separated into stipes and fronds, dried under controlled conditions and analysed for total ash, crude protein, iodine, mannitol, laminarin and alginic acid.

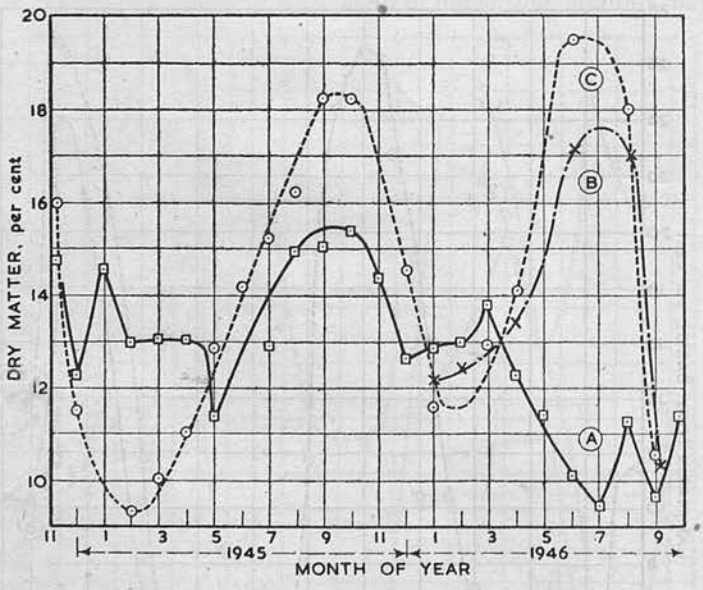

FIG. 12. *Seasonal variation in dry matter in* L. saccharina (*sea loch*).
A. *In the stipes.* B. *In the whole plant.* C. *In the fronds*

The results have all been calculated on the anhydrous basis but graphs are given of the variation in the dry matter content enabling any result to be calculated out on the wet plant.

The factors affecting the composition which have been studied are (*a*) season of the year, (*b*) locality and (*c*) depth of immersions of the weed.

The variations in composition have been found to be due almost entirely to variations in the composition of the fronds.

Laminarin, a glucose polymer, which may be formed under the same conditions as starch in the land plants, has been found to occur only in the fronds. It is absent from the annual *saccorhiza bulbosa*, samples of which have been analysed during the two summers.

Considering the number of variables which might affect the chemical composition of algae and the fact that samples were taken at random irrespective of their age, one would not be surprised to find appreciable differences in the composition in different years. The results, however, show that with one or two exceptions, the composition is relatively the same at the corresponding season of each year.

It is perhaps worth recording that the summer of 1945 was dry and very sunny while that of 1946 was cloudy and wet with very little sunshine.

The investigation is continuing.

Acknowledgement

The work described in these reports forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association.

The author wishes to thank Miss M. Dunne and Mr. P. A. Gower for assistance with the analytical work and the Association for permission to publish.

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Musselburgh, Midlothian Received December 15, 1947

[1] Black, W. A. P., J.S.C.I. 1948, 67, 165
[2] *Ibid*, 169
[3] Spence, M., J. Bot. 1918, 56, 281, 337
[4] Ricard, R., Bull. Soc. Chim. biol. 1931, 13, 417
[5] Tessler, Marine Products of Commerce
[6] Fritsch, F. E., Structure and Reproduction of the Algae. Vol. II, p. 207

Reprinted from the Journal of the Marine Biological Association of the United Kingdom, Vol. xxix, September 1950, pages 379–387
PRINTED IN GREAT BRITAIN

THE SEASONAL VARIATION IN THE CELLULOSE CONTENT OF THE COMMON SCOTTISH LAMINARIACEAE AND FUCACEAE

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(Text-figs. 1–6)

Introduction

Algal cellulose appears to have been relatively little studied and there is no information as to its seasonal variation in the brown marine algae. A study of the literature reveals numerous contradictions. In 1884, Stanford indicated in a report on alginic acid that he had obtained pure cellulose representing 10–15% of the air-dried plant.

Kylin (1915) showed that algal cellulose gave the characteristic colour reaction with iodine and sulphuric acid. He boiled the material in turn with 1·25% sulphuric acid and 1·25% sodium hydroxide and called the insoluble residue cellulose.

In 1926, Atsuki & Tomodo obtained a crude fibre figure of 6% for *Laminaria saccharina*, but stated that the greater part of the crude fibre of the laminarias consisted of the hemicelluloses and that there was so far no evidence of the normal cellulose.

Ricard (1931) removed the alginic acid with sodium carbonate, then treated the residue with dilute boiling solutions of sulphuric acid and potassium hydroxide. The hydrolysis of the residual material with sulphuric acid gave only traces of reducing sugars, and the author did not regard it as cellulose but called it algulose. *L. flexicaulis* contained 4·3–7·6% of this material and *L. saccharina* 2·8–10·9%.

Dillon & O'Tuama (1935), however, isolated cellulose from the brown algae. The residue after the removal of alginic acid with dilute ammonia was treated with dilute hydrochloric acid and then several times with boiling 5% sodium hydroxide. It was finally washed with alcohol and ether, and was shown to have the properties of cellulose. On hydrolysis with hydrochloric acid it gave glucose characterized by the isolation of the glucosazone, and with carbon disulphide it formed a viscose resembling the viscose from ordinary cellulose. They also prepared acetylated and methylated compounds with properties similar to those of the corresponding derivatives of ordinary cellulose.

Russel-Wells (1934) determined the crude fibre content of various algae and showed by its solubility in cuprammonium hydroxide and by its acetate that this fibre was cellulose.

Viel (1939) carried out a systematic study of the celluloses from *Fucus vesiculosus* (2·10%), *F. serratus* (2·81%), *Laminaria saccharina* (6·9%) and *L. cloustoni* (5·04%). Analysis showed the carbon-hydrogen percentages to be remarkably similar and in agreement with $(C_6H_{10}O_5)_n$. Hydrolysis gave 90% glucose, while thermal fractionation of the products of pyrogenation gave graphs comparable with those of previously studied vegetable celluloses.

Recent work by Percival & Ross (1949) has shown conclusively that algal cellulose is essentially the same as cotton cellulose. Hydrolysis with 72% sulphuric acid gave only D-glucose. Cellobiose octa-acetate was prepared by acetolysis indicating the presence of 1:4-β-linkages. Determinations by periodate oxidations indicated a chain length of 160 units, but the original cellulose was no doubt degraded during isolation. Finally, X-ray diagrams of algal cellulose showed the characteristic pattern of normal cellulose.

Experimental

The samples analysed were those taken in 1946 and previously reported by the writer (1948*a*,*b*, 1949). The cellulose was estimated as follows.

Ground seaweed (2 g.) was boiled under reflux with sulphuric acid (200 ml., 1·25%) for 30 min., filtered by suction through a 1 × 2 Gallenkamp sintered glass crucible, washed free from acid with water, and the residue boiled with sodium hydroxide (200 ml., 1·25%). After boiling in this way for 30 min., the solution was filtered through the 1 × 2 crucible and washed with water. The residue was removed from the crucible and kept overnight in chlorine water (100 ml. saturated), filtered on a weighed hardened paper, washed free from chlorine and washed on the filter with hot sodium hydroxide solution (50 ml. N/10). The residue was washed with water until free from alkali and finally with alcohol and ether, and then dried at 50° for 10 min. to give a white product which was weighed.

When applied to *Fucus* spp. the residue after treatment with boiling sodium hydroxide and chlorine water was often slimy and difficult to filter, and separation by centrifuging was necessary.

The results are given in Figs. 1–6.

Discussion of Results

General

Very little is known regarding the structure of the cell wall of the Phaeophyceae. Fritsch (1945) concludes that it consists of pectic substances with a layer giving the reactions of cellulose adjacent to the protoplast. In the brown algae the pectic substance is no doubt alginic acid which, with its long

chain structure, will give flexibility to the plant. To withstand wave-action such a structure is probably reinforced with cellulose, and the results of this investigation show that the cellulose content increases with the depth of immersion, when additional strength is required by the plant.

Very little is known, also, regarding the metabolism of the brown algae, but in the absence of any reducing sugars, it would appear that mannitol is probably the primary product of photosynthesis. In 1933, Khouvine showed that cellulose could be synthesized from mannitol by *Acetobacter xylinum*, and quite independently Barsha & Hibbert (1934) obtained the same results and showed that the cellulose membranes were chemically identical with cotton cellulose. In the brown algae, therefore, mannitol is probably the precursor of the cellulose.

The Laminariaceae

Laminaria cloustoni

In Fig. 1 the graphs for the stipe, the frond and the whole plant show that the cellulose content on the anhydrous basis exhibits two maxima and two minima in the year. Similar graphs have been obtained for several of the other constituents (Black, 1948*a*,*b*, 1949) and for the diatom periodicity, and sometimes there is a correlation with the nutrient content of the water and the periods of rapid and slow growth (Black & Dewar, 1949).

In March, when the new frond is forming, the cellulose is at a maximum of 5·76% in the frond and 10·27% in the stipe. From March to June/July a period of rapid growth occurs with an increase in the mannitol content while the cellulose content decreases. This may be due to the accumulation of mannitol in the whole plant or to elongation during rapid growth. From June to August/September during the period of slow growth, when the nutrients are absent from the sea water, the cellulose content increases again, while at the same time laminarin is also increasing (Black, 1948*a*). In September, when the nutrients are again regenerated in the sea water, there is probably a second period of growth and a decrease in the cellulose content occurs. It would appear, therefore, that when the results are expressed on the anhydrous basis a correlation exists between the cellulose content and the periods of rapid and slow growth. When the results, however, are calculated on the wet basis (Fig. 2) two maxima are again recorded, one in February/March and the other from September to November. On the dry basis the cellulose content begins to fall in April and continues to fall until June; on the wet basis it is constant and at a minimum from April to June, due to the dry weight increasing with a rapid increase in mannitol. From June to November the cellulose on the wet basis increases while on the dry basis it begins to decrease in September, the increase in dry-weight content now being due to an increase in laminarin. A rapid decrease then occurs in December, the period of sporogenesis of this species, and this is probably accompanied by an increase in the uptake of water, as there is a considerable decrease in the dry-matter content both in

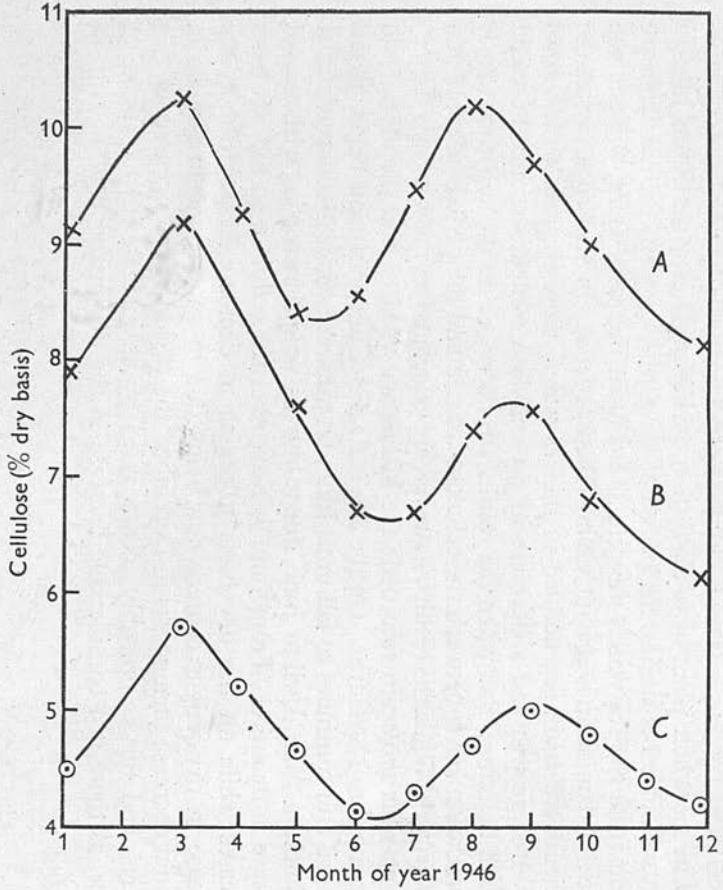


Fig. 1. Seasonal variation in the cellulose content of *Laminaria cloustoni* (dry basis): (*A*) in the stipes; (*B*) in the whole plant; (*C*) in the fronds.

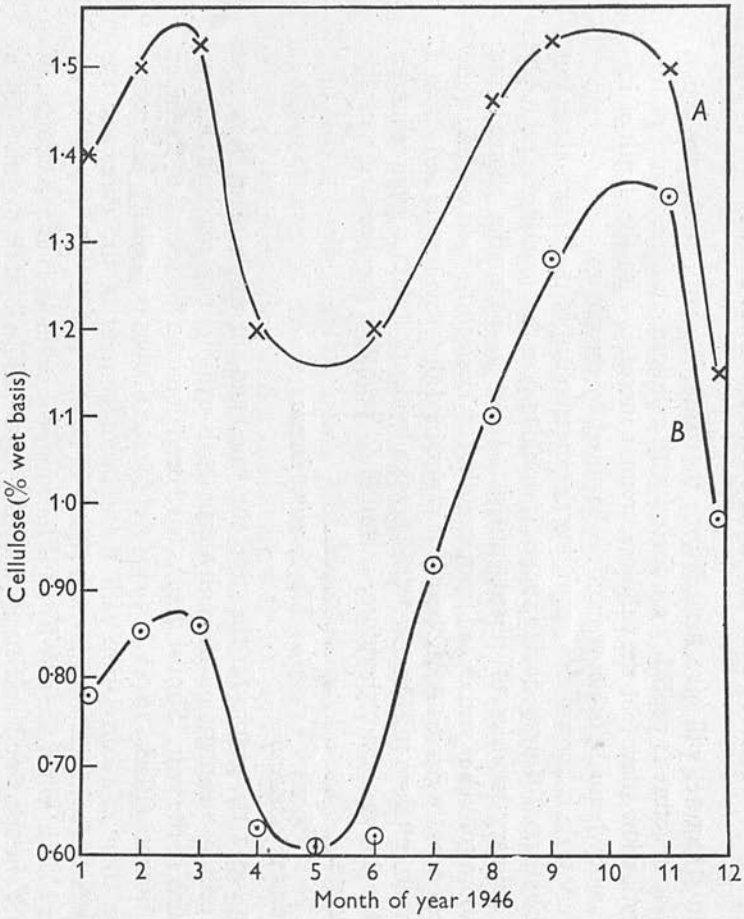


Fig. 2. Seasonal variation in the cellulose content of *Laminaria cloustoni* (wet basis): (*A*) in the stipes; (*B*) in the fronds.

stipe and frond, which explains the rapid decrease on the wet basis while there is little change on the dry basis. On the wet basis, therefore, there would appear to be a correlation between the cellulose content and the reproductive cycle of the plant.

Laminaria saccharina

In this species the cellulose content (dry basis) of the frond (4–5%, Fig. 3) is of the same order of magnitude as that of *L. cloustoni* frond (Fig. 1), but the stipes contain less cellulose (7–8%) than those of *L. cloustoni* (8·5–10%). Considerably less seasonal variation occurs in this species, but despite this the two maxima are apparent in the year and occur at approximately the same time as those of *L. cloustoni*.

Laminaria digitata.

The cellulose content of this species (Fig. 4) undergoes a similar seasonal variation to that of *L. saccharina*, with the exception of the loch frond samples in which the variation is between 3 and 5%. In general, however, the graphs are very similar and exhibit two maxima in the year and a distinct minimum in July.

The cellulose graphs on a wet basis for *L. saccharina* and *L. digitata* have been omitted. The only outstanding feature in both species is that the graphs for the open-sea samples show less seasonal variation (0·5–0·7%) than those for the loch samples (0·5–1·0%). This is due to the marked seasonal variation in dry matter which occurs in the loch samples as the result of the accumulation of laminarin. This is in agreement, to a certain extent, with the theory that the cellulose content can be correlated with the periods of growth. At Atlantic Bridge the *L. digitata* frond grows to a length of 7–8 ft. (213–244 cm.), growth appears to continue throughout the summer and there is very little laminarin formed. The dry weight shows little change and there is no marked variation in the cellulose content. On the other hand, in Loch Melfort, the *L. digitata* frond only grows to a length of about 2–3 ft. (61–91 cm.), laminarin accumulates and there is a marked increase in the dry-matter content. It appears, therefore, that laminarin is formed when photosynthesis can proceed, but some other factor is limiting growth.

The Fucaceae

With the exception of *Fucus spiralis*, which contains approximately 4·5% of cellulose (dry basis) throughout the year, there appears to be a correlation between the cellulose content of the Fucaceae and the depth of immersion of the species, the cellulose increasing from about 1% in *Pelvetia canaliculata* to 2–2·5% in *Fucus serratus* and *F. vesiculosus*. On the anhydrous basis the cellulose content of *Pelvetia canaliculata*, *Ascophyllum nodosum* and *Fucus spiralis* remains relatively constant throughout the year (Fig. 5). But when the results

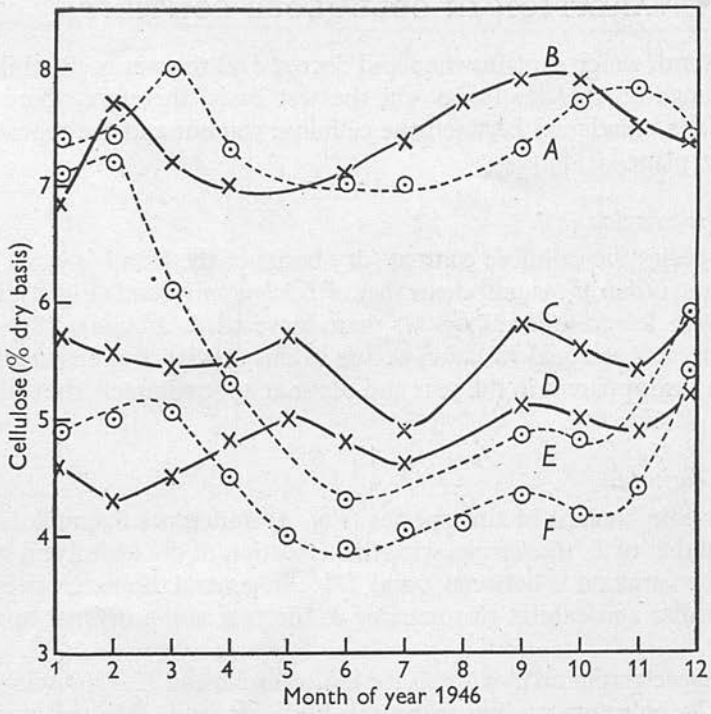


Fig. 3. Seasonal variation in the cellulose content of *Laminaria saccharina* (dry basis): (*A*) in the open-sea stipes; (*B*) in the loch stipes; (*C*) in the loch whole plants; (*D*) in the loch fronds; (*E*) in the open-sea whole plants; (*F*) in the open-sea fronds.

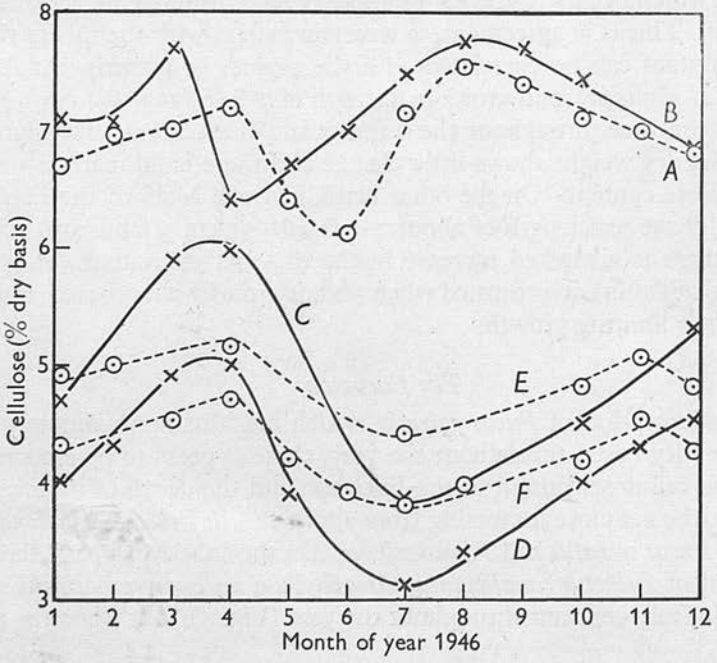


Fig. 4. Seasonal variation in the cellulose content of *Laminaria digitata* (dry basis): (*A*) in the open-sea stipes; (*B*) in the loch stipes; (*C*) in the loch whole plants; (*D*) in the loch fronds; (*E*) in the open-sea whole plants; (*F*) in the open-sea fronds.

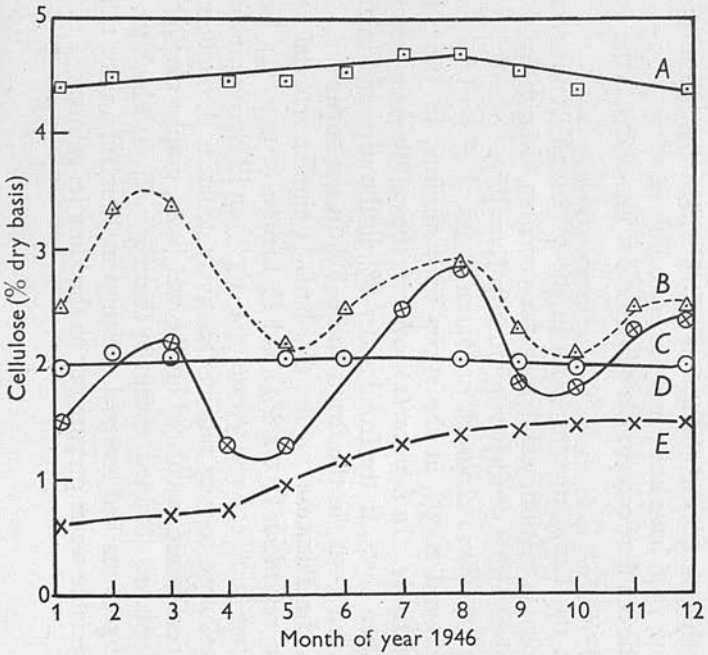


Fig. 5. Seasonal variation in the cellulose content of the Fucaceae (dry basis): (*A*) *Fucus spiralis*; (*B*) *F. serratus*; (*C*) *F. vesiculosus*; (*D*) *Ascophyllum nodosum*; (*E*) *Pelvetia canaliculata*.

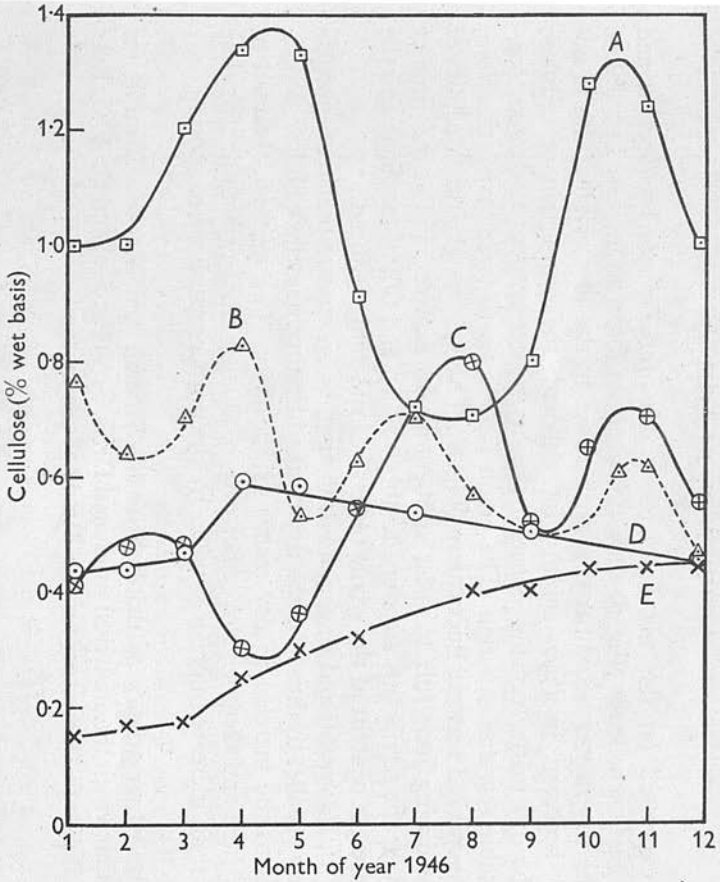


Fig. 6. Seasonal variation in the cellulose content of the Fucaceae (wet basis): (*A*) *Fucus spiralis*; (*B*) *F. serratus*; (*C*) *F. vesiculosus*; (*D*) *Ascophyllum nodosum*; (*E*) *Pelvetia canaliculata*.

are expressed on the wet basis the graphs for *Pelvetia canaliculata* and *Ascophyllum nodosum* (Fig. 6) show no change of form, an increase in cellulose in the dry matter accompanying an increase in the dry weight. This is not true, however, in *Fucus spiralis*, for although the proportion of cellulose in the dry matter remains constant the dry-matter content undergoes considerable seasonal variation. The graph for *F. spiralis* (wet basis), therefore, shows marked seasonal fluctuations (Fig. 6). This species takes in a quantity of water during June/July to assist in liberating its gametes, and at this time of the year the fertile tips are considerably swollen and full of mucilage and the dry-weight content of the whole plant is considerably reduced.

With *F. serratus* and *F. vesiculosus* the graphs on the wet basis are approximately parallel to those for the dry basis indicating that the cellulose contributes to the increase in dry matter of the plant. The minima obtained in May/June and October/November may be due to rapid growth at that time and/or an increased uptake of water by the plant preparatory to sporing.

The writer wishes to thank Miss B. Graham and Mr W. Cornhill for assistance with the analytical work, and Dr E. G. V. Percival and Dr A. G. Ross of Edinburgh University for the details of the method.

Summary

Monthly samples of the common British Laminariaceae and Fucaceae taken during 1946 have been analysed for cellulose.

The cellulose content has been found to increase with the depth of immersion of the weed.

In the Laminariaceae, both in the stipe and frond it undergoes marked seasonal variation, exhibiting two maxima, March/April and September/October with a distinct minimum in June/July.

In *Laminaria cloustoni*, in March, maxima of 5·7 (dry basis) occur in the fronds and 10·3% in the stipes with minima, in June, of 4·1 and 8·4% respectively. In *Laminaria saccharina* the cellulose content (dry basis) varies between 4–5% in the frond and 7–8% in the stipe and in *Laminaria digitata* between 3–5% in the frond and 6–8% in the stipe.

In the Fucaceae, on the dry basis, a similar seasonal variation occurs in *Fucus vesiculosus* (1·2–2·8%) and *F. serratus* (2·1–3·5%), but in *Ascophyllum nodosum* (2·0%), *Fucus spiralis* (4·4–4·7%) and *Pelvetia canaliculata* (0·6–1·5%) the cellulose content remains relatively constant throughout the year. When the results are calculated on the wet basis, graphs parallel to those for the dry basis are obtained, except in *Fucus spiralis*, in which the cellulose content undergoes marked seasonal variation in June/July, since this species takes in appreciable water preparatory to shedding its gametes.

REFERENCES

ATSUKI, K. & TOMODO, Y., 1926. Studies on seaweeds of Japan. First report on the chemical constitution of the Laminaria. *Journ. Soc. Chem. Ind. Japan*, Vol. 29, p. 132 B.

BARSHA, J. & HIBBERT, H., 1934. Cellulose membrane from mannitol. *Canadian Journ. Research*, Vol. 10, pp. 170–9.

BLACK, W. A. P., 1948*a*. Seasonal variation in the chemical constitution of some of the Laminariaceae common to Scotland. *Journ. Soc. Chem. Ind.*, Vol. 67, pp. 165–72.

—— 1948*b*. The seasonal variation in chemical composition of some of the littoral seaweeds common to Scotland. Part I. *Ascophyllum nodosum*. *Journ. Soc. Chem. Ind.*, Vol. 67, pp. 355–7.

—— 1949. Seasonal variation in chemical composition of some of the littoral seaweeds common to Scotland. Part II. *F. serratus*, *F. vesiculosus*, *F. spiralis* and *Pelvetia canaliculata*. *Journ. Soc. Chem. Ind.*, Vol. 68, pp. 183–9.

BLACK, W. A. P. & DEWAR, E. T., 1949. Correlation of some of the physical and chemical properties of the sea with the chemical constitution of the algae. *Journ. Mar. Biol. Assoc.*, Vol. XXVIII, pp. 673–99.

DILLON, T. & O'TUAMA, T., 1935. The cellulose of marine algae. *Sci. Proc. Roy. Dublin Soc.*, Vol. 21, pp. 147–52.

FRITSCH, F. E., 1945. *The Structure and Reproduction of the Algae*. Vol. 2, p. 24.

KHOUVINE, Y., 1933. Synthesis of cellulose from mannitol by *Acetobacter xylinum*. *Comp. Rend. Acad. Sci., Paris*, T. 196, pp. 1144–6.

KYLIN, K., 1915. Biochemistry of sea algae. *Ztschr. Physiol. Chem.*, Bd. 94, pp. 337–425.

PERCIVAL, E. G. V. & ROSS, A. G., 1949. Marine algal cellulose. *Journ. Chem. Soc.*, pp. 3041–3.

RICARD, R., 1931. Nature and seasonal variation of carbohydrates in laminaria. *Bull. Soc. Chim. Biol.*, Vol. 13, pp. 417–35.

RUSSEL-WELLS, B., 1934. Cellulose in marine algae. *Ann. Bot.*, Vol. 48, pp. 435–41.

STANFORD, E. C. C., 1884. On algin. *Journ. Soc. Chem. Ind.*, Vol. 3, pp. 297–302.

VIEL, G., 1939. Sur les celluloses des algins. *Comp. Rend. Acad. Sci., Paris*, T. 208, pp. 532–4.

Reprinted from the Journal of The Science of Food and Agriculture, 1954, No. 9, pp. 445–448

THE SEASONAL VARIATION IN THE COMBINED L-FUCOSE CONTENT OF THE COMMON BRITISH LAMINARIACEAE AND FUCACEAE

By W. A. P. BLACK

In previous communications[1–3] the seasonal variation in the total ash, crude proteins, laminarin, mannitol, alginic acid, cellulose and iodine for the common British Laminariaceae and Fucaceae has been reported. The present article gives the seasonal variation for combined L-fucose in the monthly samples collected during 1946.

Introduction

Fucoidin, a polyfucose ethereal sulphate, was first described and named by Kylin,[4] who prepared it[5] from *Laminaria digitata* and showed that it contained the methylpentose, L-fucose, by isolating the phenylhydrazone on hydrolysis. Its isolation and a summary of the work that has been carried out on it are given in a recent publication.[6]

Methods of estimating fucoidin were investigated by Cameron, Ross & Percival,[7] who adopted the method described by Nicolet & Shinn[8] which made possible the determination of fucose in hydrolysed seaweed samples. The method involved the oxidation of the L-fucose with periodic acid by which the terminal $-CH(OH)\cdot CH_3$ group was converted into acetaldehyde. This was then estimated by forming the compound with sodium bisulphite and determining the amount of bisulphite used by titrating with iodine. This method gave high results, indicating that an aldehyde or ketone other than acetaldehyde or formaldehyde was liberated on treatment of seaweed hydrolysates with periodate. An improved method[9] has been worked out by which the acetaldehyde liberated is determined colorimetrically by the method of Fromageot & Heitz.[10]

Specimens of fucoidin have been isolated from seaweed samples and attempts made to purify it by Percival & Ross[11] and Conchie & Percival.[12] It has not yet been possible to obtain a sample of fucoidin that is free from traces of uronic acid, xylose and galactose, and it is not known whether these constituents are a part of the fucoidin molecule or are adventitious impurities. Consequently, the figures given in this report are for combined L-fucose and have not been converted to fucoidin. The purest sample of fucoidin so far prepared[11] gave 56·7% of L-fucose on hydrolysis (chromatographic method).

Experimental

The samples analysed were those taken in 1946 and previously reported on by the writer.[1, 2] The combined fucose was estimated by the improved method already referred to,[9] and the results for L-fucose (as $C_6H_{12}O_5$) are given in Figs. 1–5.

Discussion of results

General

Nothing is known about the part played by fucoidin in the metabolism of the brown algae. The results of the present investigation show that the fucoidin content varies from as little as 2% in the Laminariaceae to over 20% in the Fucaceae (dry basis), and that a definite correlation exists between the depth at which the alga is growing and its fucoidin content, which decreases with the depth of immersion.

It has been suggested that it is present in the intercellular mucilage and workers have isolated it from the mucilage that exudes from *Laminaria digitata* fronds[13] and from the fruiting tips of *Ascophyllum nodosum*,[14] but work (unpublished) has shown that this mucilage also contains sodium alginate and is not a good source of fucoidin. Work by F. T. Walker (unpublished), however, on the staining of sections with toluidine blue, also indicates that it is in the intercellular mucilage. It is an exceedingly hygroscopic substance and its function in the Fucaceae, particularly in the most exposed alga *Pelvetia canaliculata*, where it occurs to over 20% of the dry matter, may be to prevent desiccation on prolonged exposure.

In general, it is at a minimum in May when mannitol and laminarin are at a minimum and alginic acid and total ash are maxima. From June onwards Parke[15] has shown that there is a period of slow growth in our inshore waters, a time that coincides with the lack of nitrate in our waters. During this period the carbohydrates, mannitol and laminarin, later

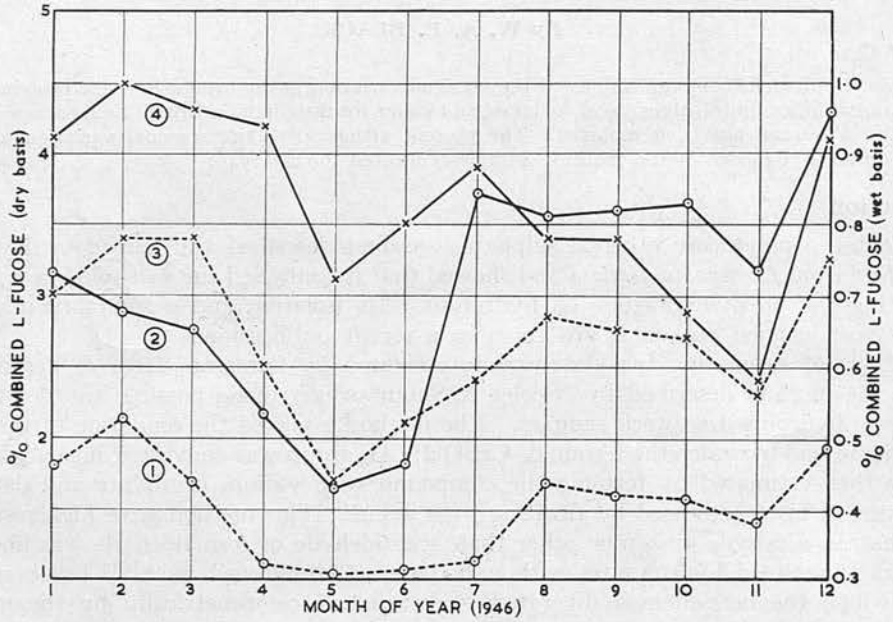


FIG. 1.—*Seasonal variation in combined* L-*fucose in* L. cloustoni

(1) Stipes (wet basis)
(2) Fronds (wet basis)
(3) Stipes (dry basis)
(4) Fronds (dry basis)

utilized in sporing and as a food reserve for the winter months, accumulate, and this is also true for fucoidin as, in many cases, the graph is parallel to that for mannitol. It may be that fucoidin is also a food reserve substance, particularly in the Fucaceae, where laminarin, considered to be the food reserve of the Laminariaceae, is very low.

Laminaria cloustoni

In Fig. 1 the results for *L. cloustoni* frond and stipe are expressed on the dry- and fresh-weight basis and the graphs are, in general, parallel, showing that an increase in the dry-matter content is partly accounted for by an increase in fucoidin. Both on the fresh- and dry-weight basis the variations in the fucose content in the frond are reproduced in the stipe, graphs 1 and 2 and 3 and 4 being approximately parallel to each other. The L-fucose (dry basis) is at a minimum in May (3·1% in the fronds and 1·7% in the stipes) when the old frond has been cast and the remaining frond consists of new tissue. As photosynthesis proceeds, it increases to a maximum in July/August (3·9% in the fronds and 3·1% in the stipes), decreasing in November and then increasing in February to 4·5% of the dry matter of the fronds and 3·4% of the stipes. It then decreases from February to May. The graphs are very similar to those previously obtained for mannitol.[3]

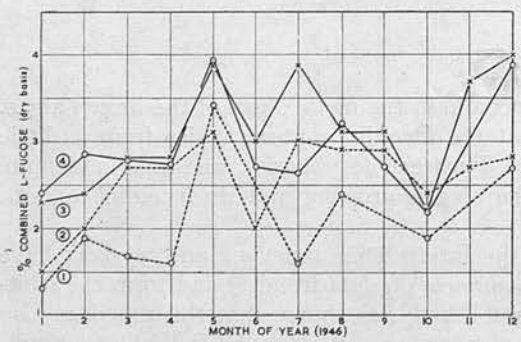


FIG. 2.—*Seasonal variation in combined* L-*fucose in* L. digitata

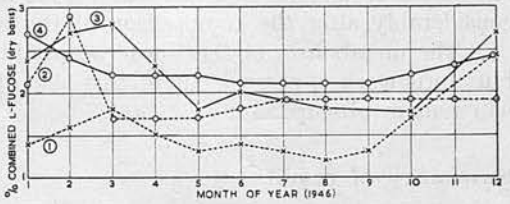
(1) Sea-loch stipes
(2) Open-sea stipes
(3) Sea-loch fronds
(4) Open-sea fronds (dry basis)

Laminaria digitata

For *L. digitata*, samples from a sea loch and an exposed position have been analysed (Fig. 2). As with *L. cloustoni* the fucose content is always higher in the frond than in the stipe and is slightly higher in the plants from the sea loch where, in general, growth is more restricted than in the exposed conditions, owing to the lower nutrient content of the water and the almost complete absence of wave action. The fucose content (1–4%) is of the same order as that of *L. cloustoni* (1–4·5%), dry basis, but the variation is more erratic. In general, the graphs follow those obtained for mannitol, particularly the loch sample where the frond graph for L-fucose is practically parallel to the mannitol graph.[3]

FIG. 3.—*Seasonal variation in combined* L-*fucose in* L. saccharina

(1) Open-sea stipes
(2) Sea-loch stipes
(3) Open-sea fronds
(4) Sea-loch fronds (dry basis)



Laminaria saccharina

In the *L. saccharina* (Fig. 3) open-sea and loch samples, very little seasonal variation occurs in the fucose content, i.e. only between 1 and 3% (dry basis).

The Fucaceae

In the Fucaceae, no recognizable seasonal variation occurs in the L-fucose content for any individual species when the results are expressed on the anhydrous basis (Fig. 4), but considerable variation occurs between the different species, the L-fucose increasing with the degree of emergence of the alga, i.e. the most exposed alga *Pelvetia canaliculata* contains up to 13% of L-fucose whereas *Fucus serratus*, growing at the low-water mark, contains only 7% (dry basis). On the wet basis (Fig. 5) considerable variation occurs,

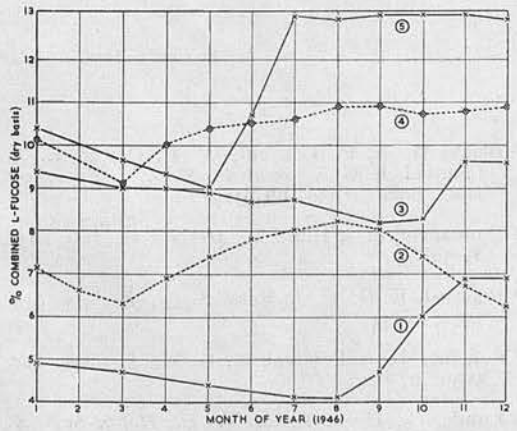


FIG. 4.—*Seasonal variation in combined* L-*fucose in*

(1) *F. serratus*
(2) *Ascophyllum nodosum*
(3) *F. vesiculosus*
(4) *F. spiralis*
(5) *Pelvetia canaliculata* (dry basis)

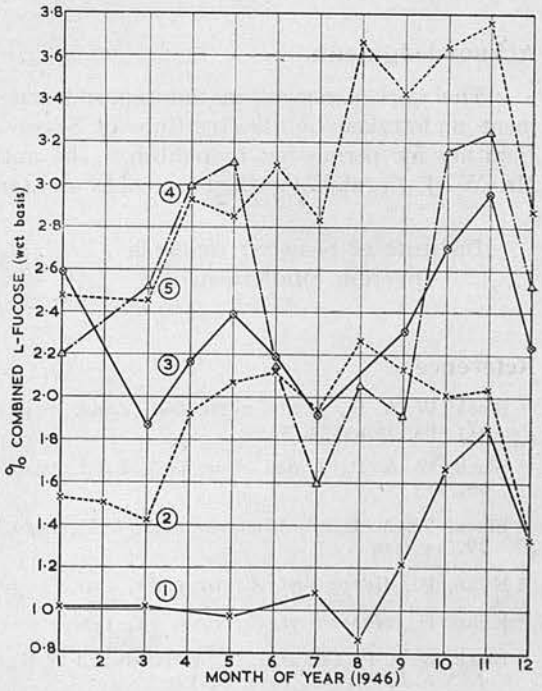


FIG. 5.—*Seasonal variation in combined* L-*fucose in*

(1) *F. serratus*
(2) *Ascophyllum nodosum*
(3) *F. vesiculosus*
(4) *F. spiralis*
(5) *Pelvetia canaliculata* (wet basis)

particularly during the months of June/July when the Fucaceae, and in particular *F. spiralis*, take in considerable quantities of water to assist in liberating their gametes. At this time the fertile tips are considerably swollen and full of mucilage, which other workers have found to be a good source of fucoidin. With *F. spiralis*, for example, the L-fucose content is 10·5% of the dry matter in May and July but the dry-matter content decreases from 30% in May to 15% in July. The decrease in the L-fucose content on the wet basis from 3·1 to 1·6% can be accounted for by the uptake of water by the alga preparatory to sporing.

The Fucaceae, in general, do not undergo such rapid growth as the completely submerged Laminariaceae and are not, therefore, so subject to the marked seasonal variations in chemical composition that are the result of the periods of rapid and slow growth which occur in the sea. The uptake of water by the Fucaceae before sporing is a factor, however, which does considerably alter the composition of the wet plant.

The metabolism of the Fucaceae would appear to differ from that of the Laminariaceae, but until work is carried out in this field the function of fucoidin in the plant's metabolism will remain obscure.

Summary of results

Monthly samples of the common British Laminariaceae and Fucaceae taken during 1946 have been analysed for combined L-fucose.

The L-fucose content has been found to decrease with the depth of immersion of the weed from 13% in *Pelvetia canaliculata* to less than 4% in the Laminariaceae (dry basis).

In the Laminariaceae, the fucose content is always higher in the frond than in the stipe and variations in the frond are reproduced in the stipe. In general, fucose is at a minimum in March/April and accumulates as photosynthesis proceeds in much the same way as mannitol.

In the Fucaceae, the fucose content shows no regular seasonal variation on the dry basis, but considerable differences occur between the species. On the wet basis, marked seasonal variations occur, mainly owing to the uptake of water by the plant before sporing.

Acknowledgments

The work described in this report forms part of a programme of research and development undertaken by the Institute of Seaweed Research and the author is indebted to the Institute for permission to publish. The author also wishes to thank Miss B. Graham and Mr. W. J. Cornhill for their valuable assistance with the experimental work.

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Received 5 April, 1954

References

[1] Black, W. A. P., *J. Soc. chem. Ind., Lond.*, 1948, **67**, 165, 169, 172, 355

[2] Black, W. A. P., *J. Soc. chem. Ind., Lond.*, 1949, **68**, 183

[3] Black, W. A. P., *J. Mar. biol. Ass. U.K.*, 1950, **29**, 45, 379

[4] Kylin, H., *Hoppe-Seyl. Z.*, 1913, **83**, 171

[5] Kylin, H., *Hoppe-Seyl. Z.*, 1915, **94**, 337

[6] Black, W. A. P., Dewar, E. T., & Woodward, F. N., *J. Sci. Fd Agric.*, 1952, **3**, 122

[7] Cameron, M. C., Ross, A. G., & Percival, E. G. V., *J. Soc. chem. Ind., Lond.*, 1948, **67**, 161

[8] Nicolet, B. H., & Shinn, L. A., *J. Amer. chem. Soc.*, 1941, **63**, 1456

[9] Black, W. A. P., Cornhill, W. J., Dewar, E. T., Percival, E. G. V., & Ross, A. G., *J. Soc. chem. Ind., Lond.*, 1950, **69**, 317

[10] Fromageot, C., & Heitz, P., *Mikrochem. Acta*, 1938, **3**, 52

[11] Percival, E. G. V., & Ross, A. G., *J. chem. Soc.*, 1950, p. 717

[12] Conchie, J., & Percival, E. G. V., *J. chem. Soc.*, 1950, p. 827

[13] Lunde, G., Heen, E., & Öy, E., *Hoppe-Seyl. Z.*, 1937, **247**, 189

[14] Dillon, T., Kristensen, K., & O hEochdha, C., *Proc. R. Irish Acad.*, 1953, [B] **55**, 189

[15] Parke, M., *J. Mar. biol. Ass. U.K.*, 1948, **27**, 651

Printed in Great Britain by Butler & Tanner Ltd., Frome and London

Reprinted from the Journal of the Marine Biological Association of the United Kingdom, Vol. xxix, April 1950, pages 45–72
PRINTED IN GREAT BRITAIN

THE SEASONAL VARIATION IN WEIGHT AND CHEMICAL COMPOSITION OF THE COMMON BRITISH LAMINARIACEAE

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(Text-figs. 1–20)

CONTENTS

INTRODUCTION

In previous publications the author (Black, 1948) has reported the seasonal variation in the total ash, iodine, crude protein, mannitol, laminarin and alginic acid contents of the Laminariaceae, *Laminaria cloustoni*, *L. digitata* and *L. saccharina*, for the 2-year period from November 1944 to October 1946.

The work has been continued for a further 2 years and this paper summarizes the seasonal variation in the above constituents, with the exception of iodine, for the period November 1946 to October 1948 inclusive. In addition, the seasonal variation in the fresh weight and dry weight contents has also been recorded.

The algae, in general, undergo such marked variations in chemical composition depending on the season of the year, the habitat, and the depth at which they grow, that any analysis of a sample for which the complete history is not given is of little value.

Cast weed, which usually has all the water-soluble constituents leached out either by the buffeting action of the waves before it is finally deposited on the beach, or by rain while exposed on the shore, may also have undergone appreciable bacterial decomposition, giving a false picture of the composition of the living plant. It is quite possible, too, that the time which elapses between the collection and drying may influence the composition, so that this time, together with the conditions of drying, should also be recorded.

The present investigation has shown that the method of sampling adopted is relatively sound, and that at any one time surprisingly small differences occur in the composition of individual plants taken from the same habitat, at the same depth, and dried under identical conditions.

The investigation is unique in that the method of sampling prevented the weed from coming into contact with rain, etc., which would affect its composition, and the plants were immediately dried under controlled conditions to reduce any chemical and biological changes to a minimum. Previous workers, on the other hand, do not state how their samples were collected, whether the weed was cut, or whether the samples were those of drift weed, and in what manner their samples were dried.

Although Stanford (1883, 1884*a*, *b*, 1886) in the second half of the nineteenth century carried out the pioneer work and laid the foundations for an industry based on the organic constituents of seaweed, it was not until 1919 that Lapicque carried out the first systematic investigation into the seasonal variation in the chemical composition of the marine algae. During the first world war, however, the demand for potash led to an intensive investigation being carried out by Hendrick (1916), but he was chiefly concerned with the analysis of the mineral matter of the algae.

From 1927 to 1929, Colin & Ricard (1929, 1930) collected monthly samples of *L. flexicaulis* and *L. saccharina* and examined them for dry weight, ash, mannitol, laminarin, algin and cellulose. Lunde (1937), investigating the possibilities of the seaweed off the coast of Norway as a source of raw material, collected monthly samples of *L. digitata* from 1935 to 1937 and analysed them for ash, mannitol, alginic acid, laminarin, fucoidin and nitrogen.

Similar investigations have been carried out in Japan by Atsuki & Tomoda (1926*a*, *b*), in Russia by Kizevetter (1938) and Vedrinskii (1938), and in Eire by Dillon (1943). Our knowledge, however, of the organic constituents of the marine algae is still incomplete. Accurate methods of analysis have been devised for the estimation of mannitol, laminarin, alginic acid and cellulose, while work is continuing on a method for the estimation of fucoidin. The fats and pigments require further investigation. Their nature and relative proportions in the algae are in many cases indeterminate.

No work appears to have been carried out on the algal proteins.

Minor constituents such as fucosterol and the neutral oils are at present being investigated, and it may well be that other valuable constituents have yet to be identified.

The work described in this paper forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association.

The author wishes to thank Miss B. Graham and Mr W. Cornhill for assistance with the analytical work and the Association for permission to publish.

Investigations on separate constituents

In a brief review it is impossible to summarize all the work which has been carried out on the known constituents of the brown algae. The inorganic and several of the organic constituents have attracted the attention of numerous investigators, but it is unfortunate that often the results are somewhat conflicting.

Total ash

The total ash consists of inorganic salts, presumably in solution in the cell sap, and the cations combined with the organic constituents such as alginic acid and fucoidin. In addition, the ash figure contains salts from the sea water retained on the surface of the plant. The plants were only allowed to drip before drying, since it was not considered advisable to wash off this surface water with distilled water which might affect the composition. Until about 1850 the ash of seaweeds, chiefly of the fucoid types, was the sole source of alkali for the soap and glass industries. This kelp industry was then threatened by the Le Blanc process, but as the use of iodine in medicine developed, a new kelp industry arose based on the ash of the laminarias; but this industry is also obsolete. Any future industry, however, based on seaweed will most likely depend on its organic constituents, which are mainly polysaccharide in character.

Trace elements

The accumulation of trace elements in marine algae, which can be explained by considering alginic acid as an ion-exchange material, has been studied by Cornec (1919), Vernadskii (1930), Jones (1922), Öy (1940), and Wilson & Fieldes (1941).

A study of the trace elements present in the Laminariaceae and Fucaceae common to Scotland is at present being carried out and the results will be published in a future communication.

Mannitol

Mannitol, a hexahydric alcohol common to all brown seaweeds, was first detected in *L. saccharina* by Stenhouse (1844). It appears to be the primary product of photosynthesis, previous workers having found only traces, or the complete absence, of free-reducing sugars. Like the mineral matter, the mannitol is probably all in solution in the cell sap, for if the living plant is put into distilled water the salts and mannitol rapidly diffuse through the cell wall into the surrounding water.

Although there is a recent patent by Berk (1940) on the extraction of mannitol from seaweed, the mannitol of commerce is obtained from manna, or is prepared synthetically by the catalytic reduction of fructose.

Laminarin

Laminarin has been the subject of investigation by various workers. It was first described by Schmiedeberg (1885), who isolated it from the Laminariaceae. It has since been studied by Krefting & Torup (1909), Kylin (1913), Gruzewska (1923), Colin & Ricard (1929), Lunde (1937), Nisizawa (1940), Le Gloahec & Herter (1940), and Barry (1938, 1939, 1941, 1942), and their work has been reviewed by Hassid (1944). Barry (*loc. cit.*) showed that laminarin consisted exclusively of glucose units, and that after methylation and hydrolysis it gave 2, 4, 6-trimethyl glucopyranose. He concluded that laminarin consisted of a chain of β-glucopyranose units bent into a spiral form.

Laminarin, therefore, differs fundamentally from starch and cellulose in that the glucose residues are combined by 1, 3-glycosidic linkages and not through carbon atoms 1 and 4.

The extent of oxidation of laminarin with periodic acid was used by Barry (1942) as an end group assay for this polysaccharide, and the results indicated a chain of sixteen glucose units. This chain length was not in agreement, however, with that obtained by the Haworth-Hirst method which indicated a chain length of about seventy-four glucose units.

The present investigation has shown that laminarin, present only in the frond, can be isolated in two forms, an almost water-insoluble form from *L. cloustoni*, which separates from cold water, and a more soluble form from *L. digitata*, which can only be obtained from aqueous solution on the addition of alcohol. The two forms are at present being studied.

It is not yet known what part laminarin plays in the metabolism of the algae.

Alginic acid

Alginic acid, found in all brown seaweeds, where it is believed to play an important part in the cell wall, was first isolated by Stanford (1883, 1884*a*, *b*, 1886) and has since been studied by the following workers: Hoagland & Lieb (1915), Nelson & Cretcher (1929), Bird & Haas (1931), Dillon & McGuinness (1931), Gomez (1933), Barry & Dillon (1935, 1936), Hirst, Jones & Jones (1939), Stewart & Lucas (1940), Speakman & Chamberlain (1944), Astbury (1945), and Wasserman (1949). It has been shown to be a polyuronide composed entirely of D-mannuronic acid.

Dillon & McGuinness (1931) believed that the alginic acid in the growing plant was combined with calcium and iron, and that desiccation destroyed the colloidal character of these compounds and rendered them insoluble. Bird & Haas (1931), on the other hand, believed that the alginic acid in the cell wall was in two forms: (*a*) a water soluble form, and (*b*) the acid in the free state. Recent adsorption experiments carried out by Wasserman (1949), however, have shown that the alginic acid occurs in the cell tissue of brown algae in the form of various metal salts, and is not present as the free acid.

Crude proteins

Except for the work of Haas and co-workers (1929, 1931, 1933, 1938), who succeeded in isolating an octapeptide of glutamic acid, no other work has been carried out on the nitrogen metabolism of the brown algae.

Cellulose

Although Kylin (1913, 1915, 1918, 1944) had shown that the cell-wall constituents of various seaweeds gave, with iodine and sulphuric acid, the characteristic blue colour of cellulose, considerable doubt existed for some time as to the occurrence of normal cellulose in marine algae. Thus Atsuki & Tomoda (1926*a*, *b*) stated that the greater part of the crude fibre of the laminarias consisted of the hemicelluloses, and that there was no evidence of the normal cellulose, while Ricard (1931), in the algae examined by him, did not obtain the characteristic reaction of cellulose, as found by Kylin. On the other hand, Naylor & Russel-Wells (1934) and Dillon & O'Tuama (1935) demonstrated the existence of normal cellulose in marine algae. Viel's (1939) survey of the literature reveals numerous contradictions, but recent work by Percival & Ross (1948*a*) has shown conclusively that the brown algae do contain cellulose which is fundamentally similar to the cellulose of the land plants.

Fats

Except for the work of Russel-Wells (1932) and Takahashi and his co-workers (1933, 1935, 1939), no other work appears to have been carried out on the fats of the Laminariaceae, and there is no information on the seasonal variation. Russel-Wells (1932) showed that a correlation existed between the fatty constituents and the depth of immersion of the algae. Takahashi *et al.* have, over a number of years, studied the fats of the indigenous algae of Japan and identified the various fatty acids.

Pigments

Although an appreciable amount of work has been carried out on the pigments of the Fucaceae, those of the Laminariaceae have received very little attention, the only recent work of a systematic nature being that of Manning & Hardin (1944).

Preparation and Analysis of Samples

Each month, the samples of *L. cloustoni* were collected on the reef off Cullipool, Luing Island, in approximately 4 m. of water (D.L.W.O.S.T.), every effort being made to take them from the same spot and at the same depth. The open sea samples of *L. saccharina* were taken at Rudh-an-Aoil, Shuna Island and the sea-loch samples at Eilean Coltair, Loch Melfort, where the plants were growing in 3–4 m. of water (L.W.). As with *L. cloustoni*, the samples were obtained by trawling a multi-pronged grapnel for 2 min., hoisting the grapnel and lifting the weed into the boat.

The open-sea samples of *L. digitata* were taken at Atlantic Bridge and the

sea-loch samples at Eilean Coltair, Loch Melfort, where the plants were growing in 1 m. of water (L.W.). The samples were taken by hand at low water, when the plants were partially exposed. With each species, twenty plants were taken, measured and weighed. Two plants were selected for dry-weight determinations, and two were chosen at random for chemical analysis. The plants, separated into stipes and fronds, were draped over racks in a heated shed and dried at a temperature of 25–35° C. for approximately 48 hr., after which they were ground in a Christy and Norris No. 8 Laboratory Mill, fitted with a $\frac{1}{64}$ in. perforated plate screen, giving a powder which practically all passed through a sieve of 90 meshes to the inch. As the analytical methods were evolved it was found that this fine state of division was essential, especially for the method of estimating alginic acid.

The methods of analysis used were those previously employed by the writer (1948).

In the preparation of the samples for analysis, changes in composition due to respiration, etc., were negligible provided drying was carried out immediately after sampling; samples dried rapidly at 80–90° C. confirmed the results obtained at the temperatures employed in this investigation. On the other hand, plants killed in boiling absolute alcohol before drying, as is the normal procedure in plant analysis, showed considerable loss of mannitol and mineral matter, but it was found possible to kill sections of the laminarias in boiling chloroform without materially affecting the chemical composition.

Results

The results, calculated on the anhydrous basis for the frond, stipe and whole plant, are given in Figs. 1–19 (pp. 53–67).

Before drying, the plants were divided into stipes and fronds which were weighed separately. From these results each constituent determined in stipe and frond was calculated for the whole plant.

Figs. 2, 8 and 12, giving the seasonal variation in the dry-matter contents of the fronds, the stipes and the whole plants, can be used to recalculate any of the results (expressed on the dry basis) on the fresh-weight basis.

In Fig. 20 (p. 68) the seasonal variation in the fresh weight of *L. saccharina* from Loch Melfort and Shuna Island is given, while the average figures for the three species are given in Table I.

Discussion

General

In general, the results agree reasonably well with those of the first 2 years investigated (Black, 1948), despite the fact that the summer of 1947 was exceptionally good with considerable sunshine, while 1948 was a very poor summer with considerable cloud and rain. Slight differences are occasionally found,

however, in the spring (March–April). At this period of the year, when a marked increase in the rate of photosynthesis occurs, the plant is actively producing a new frond, while the old frond is wearing away. The composition of the old frond (as shown in Table II) differs somewhat from that of the new frond, to which it is still attached, so that conditions such as rough weather, which influence the shedding of the old frond, will have an effect on the composition of the whole plant.

It would appear, therefore, that when the older part of the frond detaches itself it still contains mannitol and laminarin while the new growth contains no laminarin. This, no doubt, accounts for the sudden drop in laminarin in the spring. The new frond then undergoes a period of rapid growth and laminarin

TABLE I. MAXIMUM, MINIMUM AND AVERAGE WEIGHT OF PLANTS IN GRAMS

		Maximum	Minimum	Average of 450 plants
L. cloustoni	Frond	936	510	681
	Stipe	1787	681	1192
L. saccharina	Frond	1022	198	595
(open sea)	Stipe	227	85	198
L. saccharina	Frond	936	198	595
(loch)	Stipe	227	85	170
L. digitata	Frond	1901	340	965
(open sea)	Stipe	426	142	255
L. digitata	Frond	823	198	426
(loch)	Stipe	227	85	142

TABLE II. COMPOSITION OF THE OLD AND NEW FROND OF *LAMINARIA CLOUSTONI* COLLECTED AT CULLIPOOL ON 14 APRIL 1947 (DRY BASIS)

	Total ash	Mannitol	Laminarin
Old frond	38·8	4·0	2·7
New frond	36·7	8·0	Trace

is absent. In general, this polysaccharide is almost completely absent when there is rapid growth, i.e. in all the laminarias in the spring, in *L. digitata* at Atlantic Bridge for the greater part of the year, in the annual *Saccorhiza bulbosa* already reported, and in all the samples of *Macrocystis pyrifera*, *Nereocystis luetkeana* and *Lessonia flavicans* so far examined.

While the percentage of laminarin in *Laminaria cloustoni* fronds is over 30 from August to December (dry basis), in *L. saccharina* and *L. digitata* it falls rapidly in September, reaching 4·7% in *L. saccharina* (open sea), 5·9% in *L. saccharina* (loch) and 7·5% in *L. digitata* (open sea). Parke (1948, and private communication) has found that growth in *L. saccharina* and *L. digitata* continues at a greater rate during summer and autumn than in *L. cloustoni*. With *L. cloustoni* fronds there was practically no growth from July to December in the sublittoral fringe zone, which confirms the author's suggestion that laminarin is generally found when there is 'restricted growth'.

In March, therefore, we find the algae high in proteins and alginic acid with

the cell sap high in mineral matter and low in carbohydrates, which have been used up during the winter in respiration and probably in the synthesis of amino-acids. In the spring a rapid increase in the rate of photosynthesis occurs accompanied by an increase in the mannitol content and a decrease in the ash content, while rapid growth of the plant results in a decrease in the crude protein content. A decrease in alginic acid occurs as a result of this increase in mannitol. As summarized in Fig. 19, when the results are calculated on the anhydrous basis, ash, proteins and alginic acid are at a maximum and mannitol and laminarin at a minimum at the beginning of the spring, while in the autumn the reverse is true.

In the first 2 years investigated, with the exception of *L. digitata* in 1948, a break or flattening out of the mannitol graph for the fronds occurs in July–August of each year. This coincides with (*a*) a slowing up in the rate of growth, (*b*) the absence of nutrients in the waters, and (*c*) the probable period of sporogenesis in the case of *L. digitata* and *L. saccharina*, so that these factors together with light which is at its maximum intensity at this time are all contributory factors influencing the chemical composition of the algae.

Fronds *Laminaria cloustoni*

In May 1947 the dry-weight content is at a minimum of 13·3% (Fig. 2), laminarin is at a minimum of 1·0% (Fig. 1), while the total ash is at a maximum 37·6% (Fig. 1). At this period the new frond has probably taken over photosynthesis and the old frond has been cast. The new frond contains 14·2% mannitol (Fig. 3) and 11·8% crude proteins (Fig. 4), the mannitol having been at a minimum in March (6·4%) and the proteins at a maximum (15%), while the alginic acid was also at a maximum (19·3%).

As the ash falls to a minimum of 13% in September-October the dry-weight content reaches a maximum of 32% in September, the alginic acid a minimum of 8%, and the laminarin a maximum of 32·4%, the ash graph (Fig. 1) being the inverse of the laminarin graph.

In 1948, maxima and minima of the same order of magnitude occur at approximately the same periods, with the exception of mannitol which is 25% from June to August compared with a maximum of 22·9% in August 1947. In general, the laminarin, dry weight and mannitol are parallel, showing minima in the spring and maxima in the autumn, while the ash, crude proteins and alginic acid are the reverse, showing maxima in the spring and minima in the autumn.

In Fig. 6 the ash graphs for the 4 years investigated are given and show that the results are reproducible within a month, e.g. an ash content of 30% occurs in June–July in 1945, 1947 and 1948, and in July–August in 1946.

The only striking difference in the composition of the fronds during the 4 years now investigated is in the laminarin content which progressively increases each year from 29% in 1945 to 34% in 1948, while later work to be

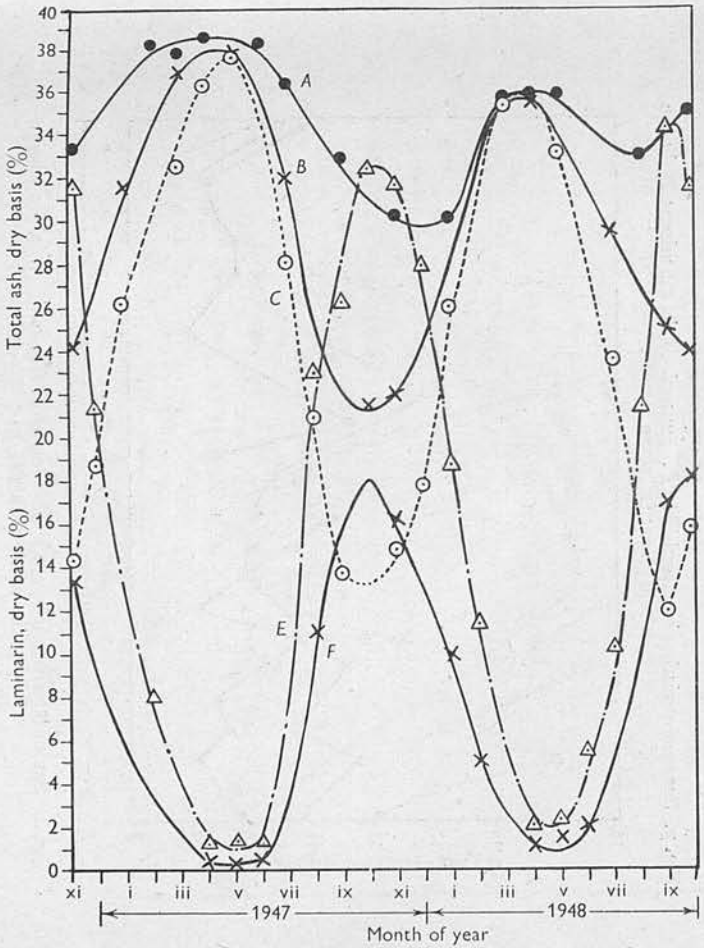

Fig. 1. Seasonal variation in total ash and laminarin in *L. cloustoni*. *A*, total ash in the stipes; *B*, total ash in whole plant; *C*, total ash in the fronds; *E*, laminarin in the fronds; *F*, laminarin in the whole plant.

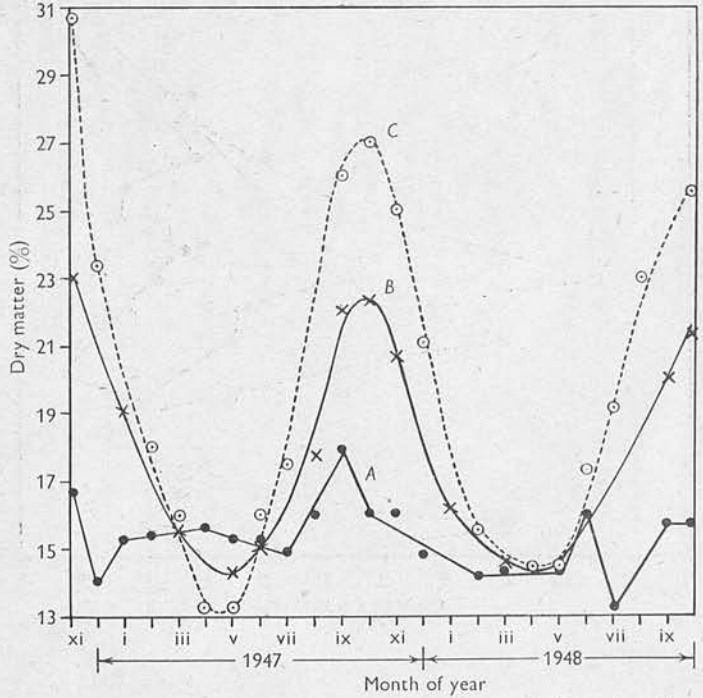

Fig. 2. Seasonal variation in dry matter in *L. cloustoni*. *A*, in the stipes; *B*, in the whole plants; *C*, in the fronds.

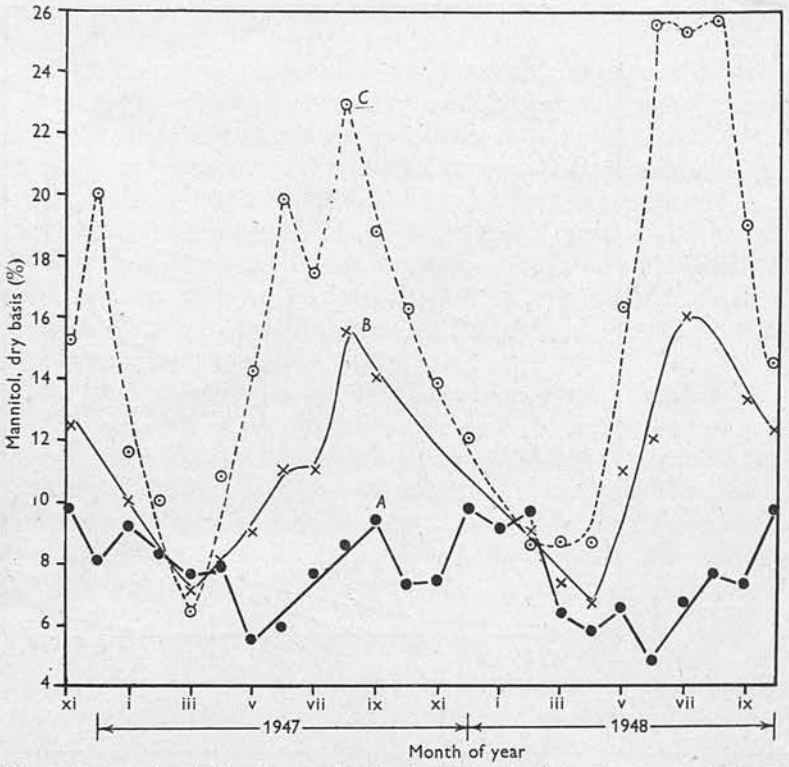

Fig. 3. Seasonal variation in mannitol in *L. cloustoni*. *A*, in the stipes; *B*, in the whole plant; *C*, in the fronds.

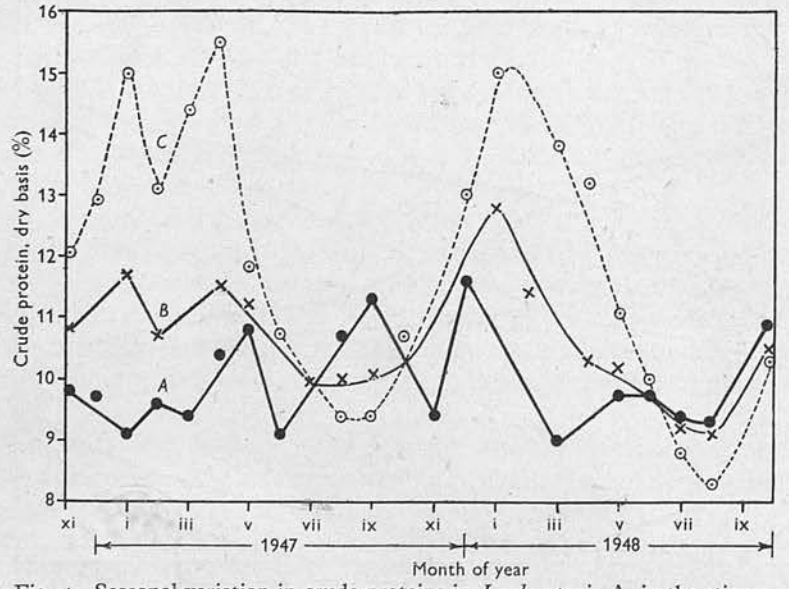

Fig. 4. Seasonal variation in crude proteins in *L. cloustoni*. A, in the stipes; *B*, in the whole plant; *C*, in the fronds.

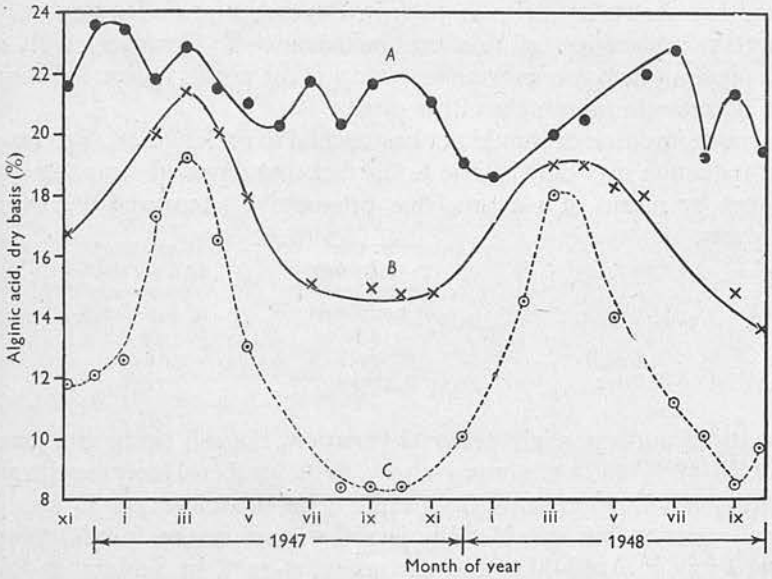

Fig. 5. Seasonal variation in alginic acid in *L. cloustoni*. *A*, in the stipes; *B*, in the whole plant; *C*, in the fronds.

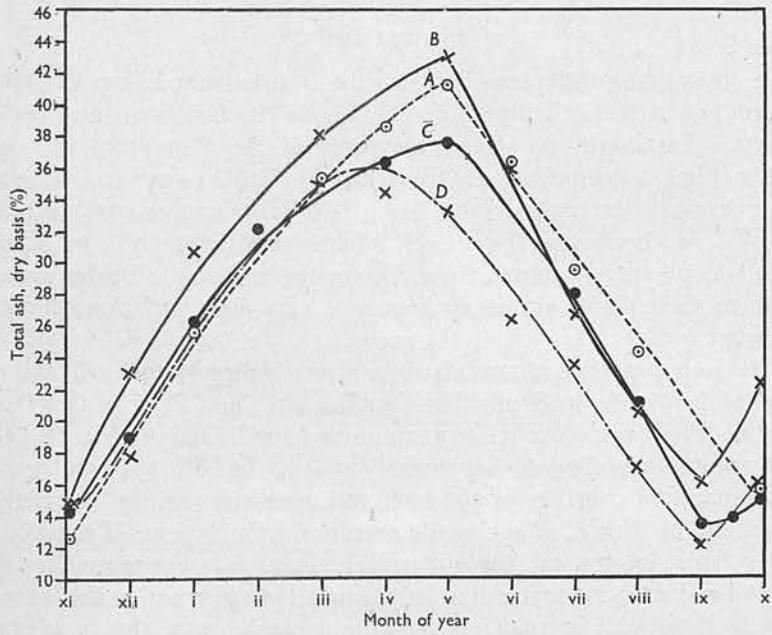

Fig. 6. Seasonal variation in total ash in *L. cloustoni* fronds. *A*, 1944–1945; *B*, 1945–1946; *C*, 1946–1947; *D*, 1947–1948.

reported has shown it to rise to 36% in December 1948–January 1949. No satisfactory explanation of this can be advanced. However, each month twenty plants of each species were weighed and the results indicate a progressive annual decrease in the weight of the plants.

Too much importance should not be attached to such figures, as the apparent weight reduction may only be due to the fact that repeated sampling from the same spot by means of a grapnel has progressively removed the larger and older plants.

	Nov. 1946 to Oct. 1947 Average weight of 240 plants (g.)	Nov. 1947 to Oct. 1948 Average weight of 240 plants (g.)
Frond	693	656
Stipe	1231	1141

Stipes

The stipes undergo slight seasonal variation, the ash being at a maximum in May (36–38%) and a minimum of 30% in November–December; in general, the variation follows that of the frond. The mannitol graph also shows seasonal variation but considerably less than that in the fronds, minima of 5% occurring in April–May and maxima of 9–10% in September–October. The dry weight content, however, shows very little variation (13–18%; Fig. 2).

The alginic acid shows no regular variation and fluctuates between 19 and 24%.

Laminaria digitata

Fronds

The outstanding difference between the fronds from Eilean Coltair (Loch Melfort) and Atlantic Bridge (open sea) is in the laminarin and dry-weight contents. Laminarin is absent for most of the year from the open-sea samples (Fig. 7), occurring only from July to October 1947 and from August to October 1948, the highest value being 15·6% in October 1948, as compared with 28% in the fronds from Loch Melfort and over 30% in *L. cloustoni* fronds. As previously stated, the environment at Atlantic Bridge favours the growth of giant plants and in consequence very little variation occurs in the dry matter content.

In the loch samples laminarin is absent from February to April, and reaches a maximum of 20% in September–October 1947 and 28% in October 1948, while the dry-weight content is at a minimum from January to March (11–12%) and a maximum in August–September (23–4%) in both 1947 and 1948.

The mannitol contents of the loch and open-sea samples are remarkably similar, and as with *L. cloustoni* the graphs are the inverse of the ash graphs (Figs. 9, 10). In general, the outstanding features are a temporary drop in mannitol and an increase in ash in July–August of 1947, and at the same period in the loch samples in 1948. A possible explanation of this is advanced in a recent publication (Black & Dewar, 1949).

The crude-protein graphs (Fig. 15) are very similar for loch and open-sea samples, exhibiting maxima of 12–14% between February and April and

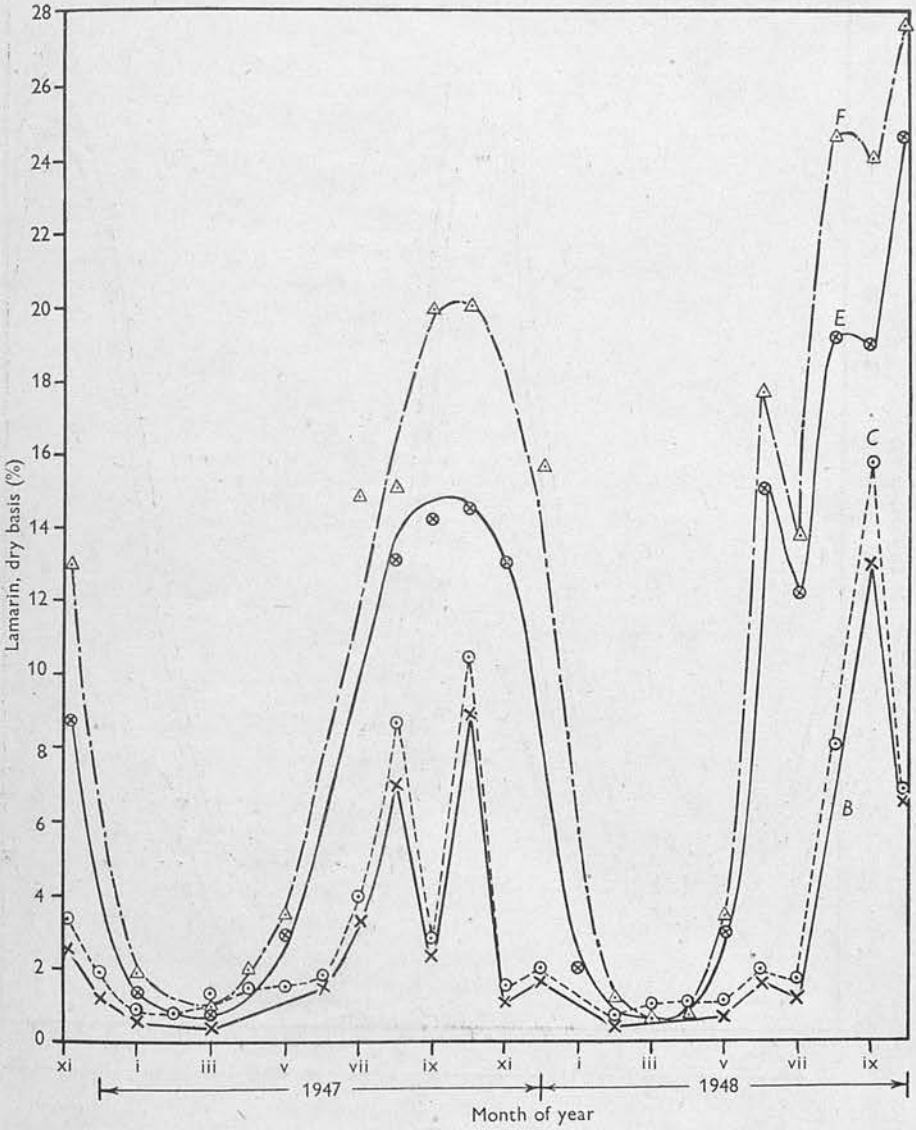

Fig. 7. Seasonal variation in laminarin in *L. digitata*. *B*, open-sea whole plant; *C*, open-sea fronds; *E*, loch whole plant; *F*, loch fronds.

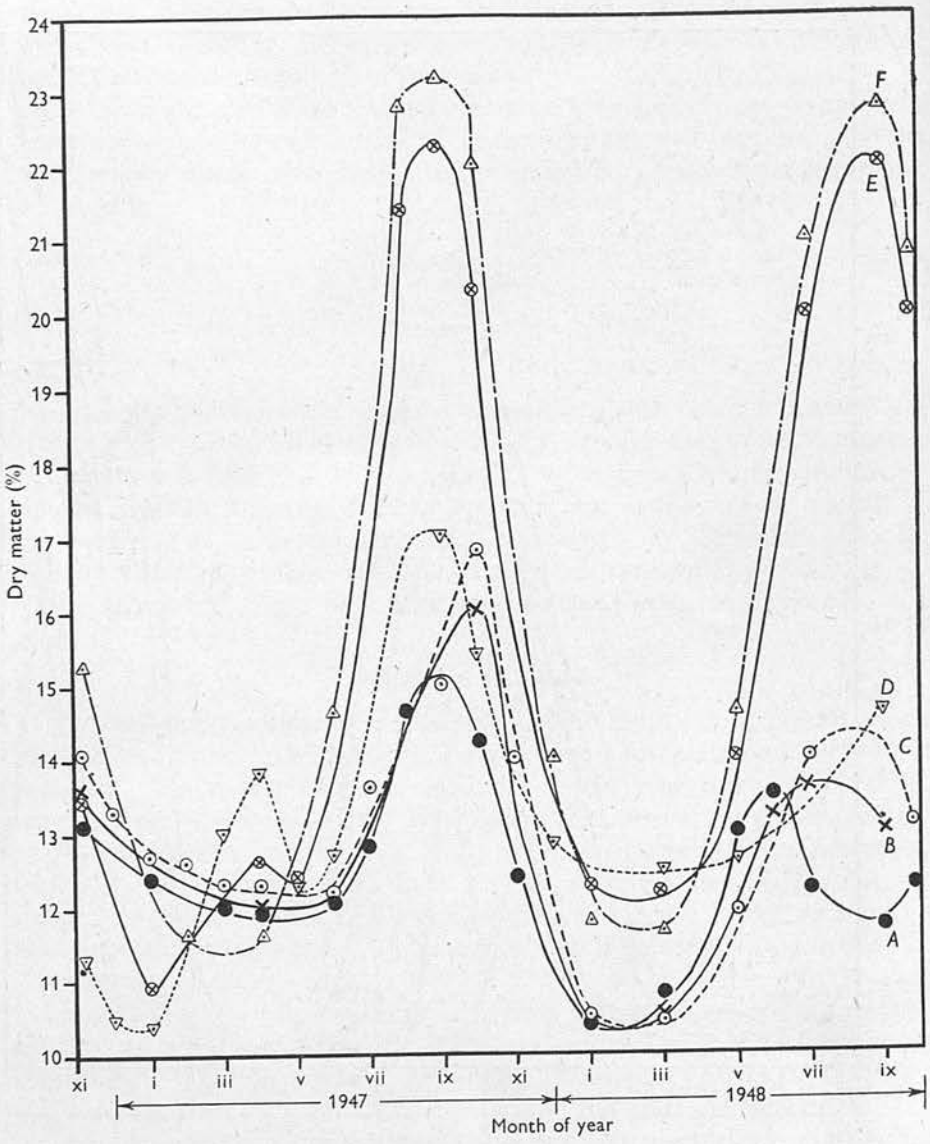

Fig. 8. Seasonal variation in dry-matter content in *L. digitata*. *A*, open-sea stipes; *B*, open-sea whole plant; *C*, open-sea fronds; *D*, loch stipes; *E*, loch whole plant; *F*, loch fronds.

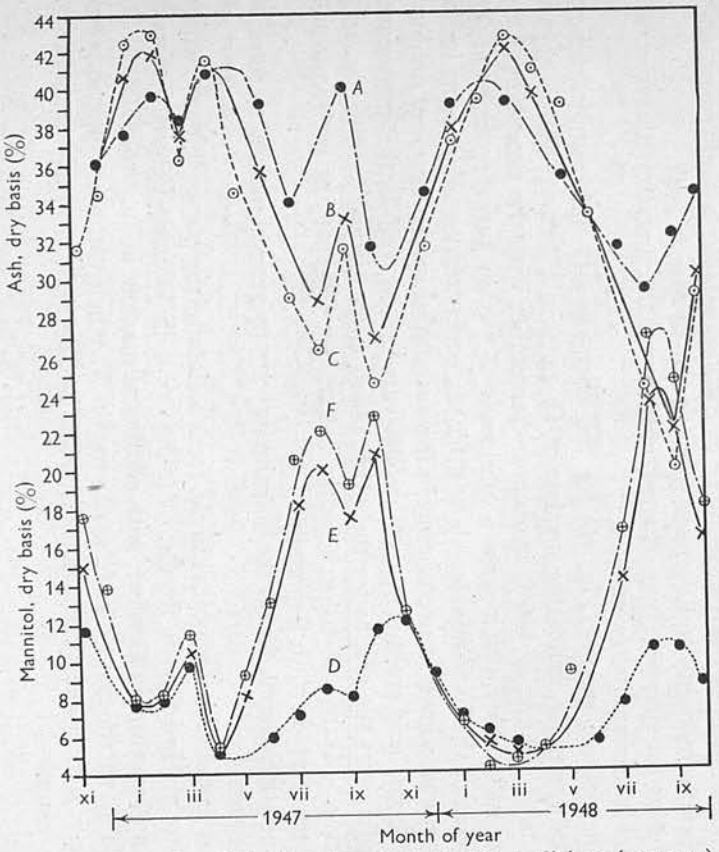

Fig. 9. Seasonal variation in ash and mannitol in *L. digitata* (open sea). *A*, ash in the stipes; *B*, ash in the whole plant; *C*, ash in the fronds; *D*, mannitol in the stipes; *E*, mannitol in whole plant; *F*, mannitol in the fronds.

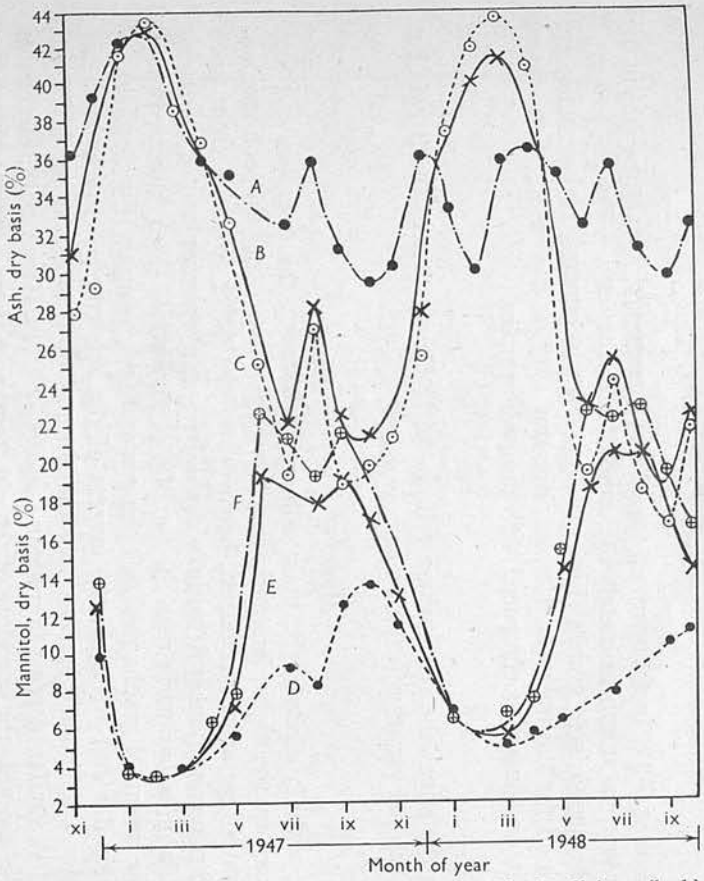

Fig. 10. Seasonal variation in ash and mannitol in *L. digitata* (loch). *A*, ash in the stipes; *B*, ash in whole plant; *C*, ash in the fronds; *D*, mannitol in the stipes; *E*, mannitol in the whole plant; *F*, mannitol in the fronds.

minima of 6–8% in the open-sea samples in August–October, and 4–6% in the loch samples at approximately the same time of the year.

The alginic acid graphs (Fig. 17) show that, in general, higher values are obtained for the open-sea samples, but this may be the result of the lower laminarin content as compared with the loch samples.

Stipes

In the stipes the composition follows fairly closely that of the fronds but the seasonal variation is within narrower limits.

Fronds *Laminaria saccharina*

As with *L. digitata*, the main differences between the open-sea and loch samples are the higher laminarin and dry-weight contents of the loch samples. As conditions at Shuna Island, however, do not differ so much from Loch Melfort as conditions at Atlantic Bridge do, the open-sea samples of *L. saccharina* contain more laminarin than the samples of *L. digitata* from Atlantic Bridge.

The mannitol graphs for the open-sea and loch samples (Figs. 13, 14) are very similar and are again the inverse of the ash graphs. In the open-sea samples a temporary increase in mannitol and drop in ash occur in March 1947. A similar increase in mannitol occurs again in March 1948, but at this time of the year the shedding of the old frond and a rapid increase in photosynthesis influence the composition. In the loch samples a similar temporary increase in mannitol and decrease in ash occur in March 1947.

During the summer (June–August) of each year the characteristic temporary decrease in mannitol occurs.

The protein graphs (Fig. 16) for the open-sea and loch samples are remarkably similar, exhibiting maxima of 13–14% in February–March of each year, and minima of 5–6% in August–September with the exception of the open-sea samples which show a minimum of 8% in July–September 1947. In general, the loch samples show a higher concentration of proteins in the spring, and a lower concentration in the autumn, than the open-sea samples. The alginic acid graphs (Fig. 18) are also very similar, and for the greater part of the 2 years the graphs for the whole plants from the two localities actually coincide. Maxima of 19–20% occur in January–February of each year and minima of 11–12% in September.

The stipe graphs for all the constituents are in general parallel to those of the fronds.

A seasonal variation occurs in the fresh weight of the plants (Fig. 20) which is most marked for the fronds. Minima occur in January–February of each year and maxima in October, with additional maxima in July 1948.

In Fig. 19, the graphs for *L. saccharina* (loch), whole plant, are superimposed and give a complete picture of the changes in composition which occur during the 2 years.

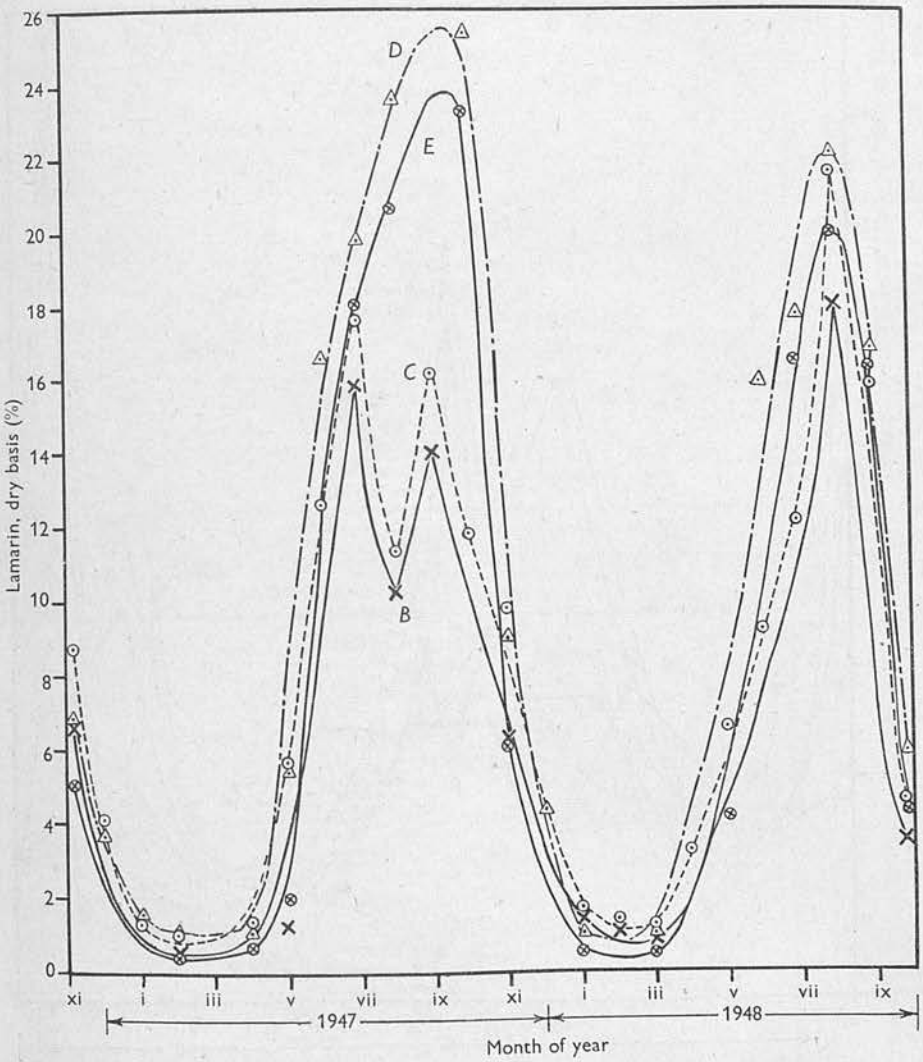

Fig. 11. Seasonal variation in laminarin in *L. saccharina*. *B*, in the open-sea whole plants; *C*, in the open-sea fronds; *E*, in the loch whole plants; *D*, in the loch fronds.

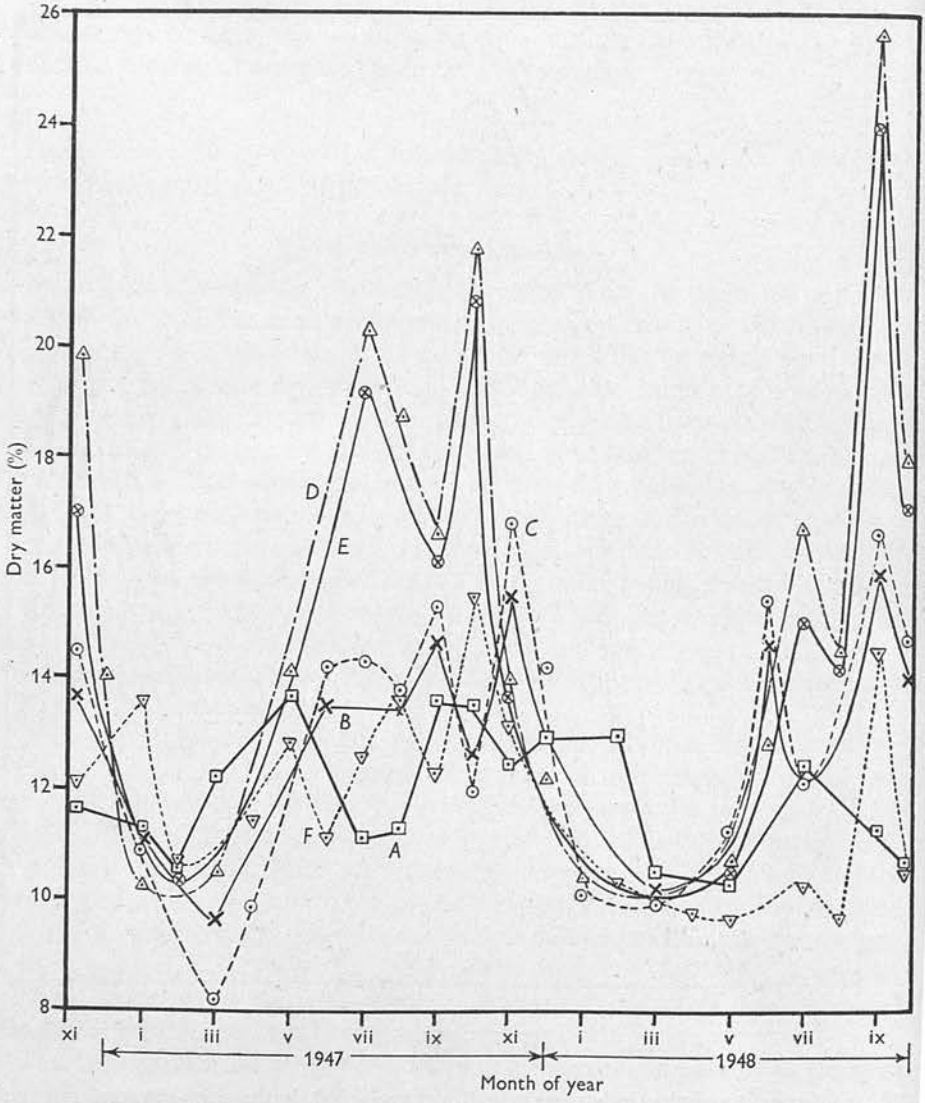

Fig. 12. Seasonal variation in dry matter in *L. saccharina*. *A*, in the open-sea stipes; *B*, in the open-sea whole plants; *C*, in the open-sea fronds; *D*, in the loch fronds; *E*, in the loch whole plants; *F*, in the loch stipes.

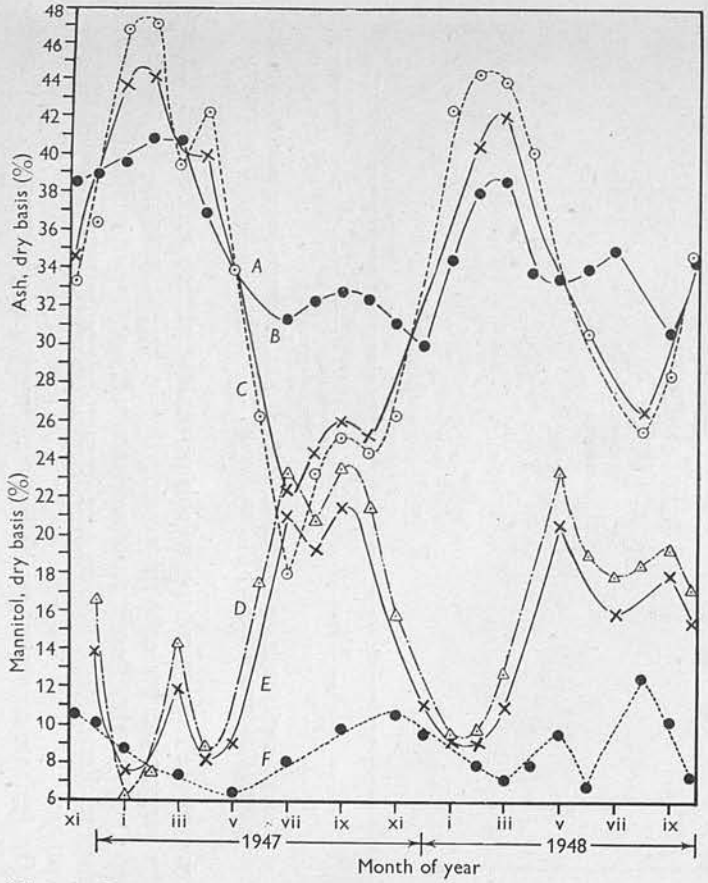

Fig. 13. Seasonal variation in ash and mannitol in *L. saccharina* (open sea). *A*, ash in the stipes; *B*, ash in the whole plant; *C*, ash in the fronds; *D*, mannitol in the fronds; *E*, mannitol in the whole plant; *F*, mannitol in the stipes.

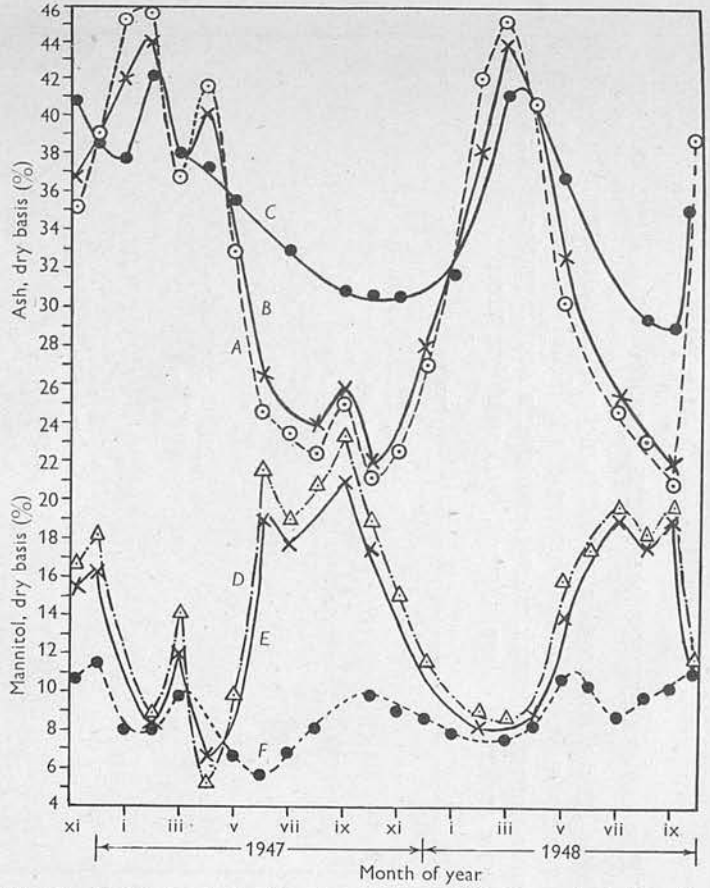

Fig. 14. Seasonal variation in ash and mannitol in *L. saccharina* (loch). *A*, ash in the fronds; *B*, ash in the whole plant; *C*, ash in the stipes; *D*, mannitol in the fronds; *E*, mannitol in the whole plant; *F*, mannitol in the stipes.

Preliminary Examination of the Haptera of the Laminarias

In Table III the composition of the haptera is compared with that of the stipe adjacent to it. With the exception of the *Nereocystis* stipe the haptera all contain a store of inorganic nitrogen which is absent from the adjacent stipes, and work at Jesus College, Oxford, by Young (private communication) has

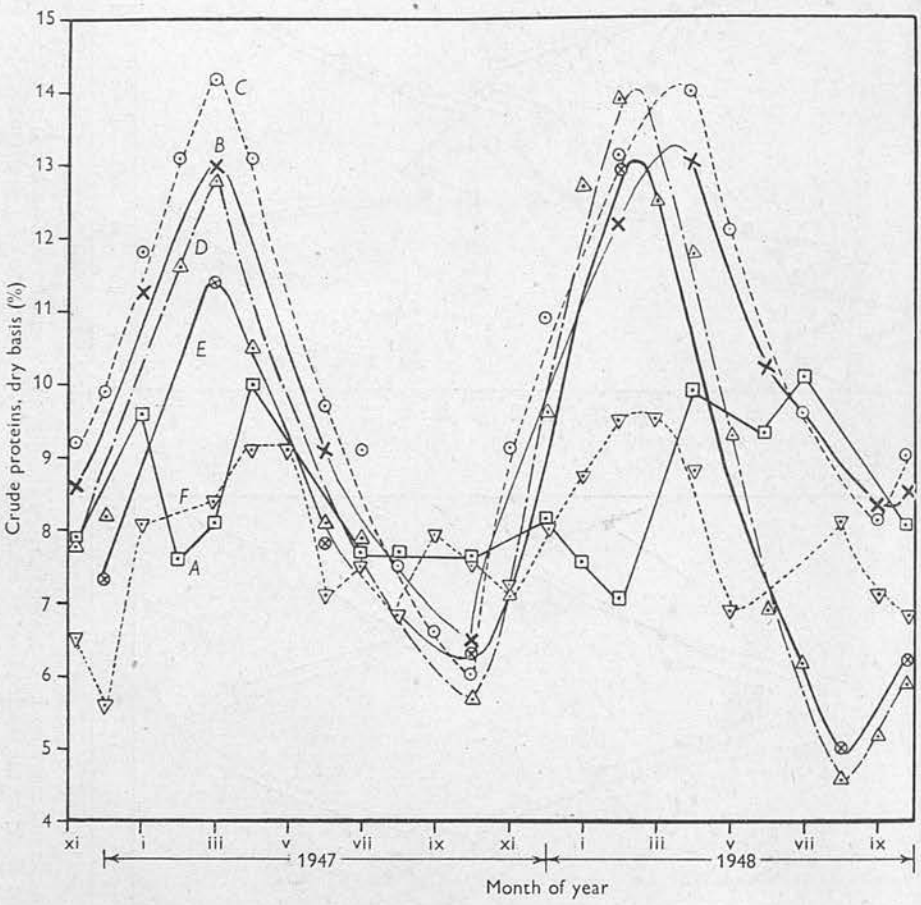

Fig. 15. Seasonal variation in crude proteins in *L. digitata*. *A*, open-sea stipes; *B*, open-sea whole plant; *C*, open-sea fronds; *D*, loch fronds; *E*, loch whole plants; *F*, loch stipes.

shown that the haptera contain a high percentage of free amino-acids. These results suggest the presence of growing tips in agreement with the result of Allsop (1948), who found an abundance of free amino-acids in actively developing tissue, but a low content in mature tissues not specially adapted for storage.

Parke (private communication) has confirmed that the haptera develop as

TABLE III. COMPOSITION OF THE HAPTERA OF THE LAMINARIACEAE *LAMINARIA CLOUSTONI*, *L. DIGITATA* AND *L. SACCHARINA*, AND *NEREOCYSTIS LUETKEANA* (DRY BASIS).

Sample	Date and place of collection	Total ash	Mannitol	Laminarin	Alginic acid	Crude proteins	In-organic nitrogen
L. cloustoni, holdfast	July 1948, Cullipool	34·1	6·0	<1·0	6·6	14·0	0·17
L. cloustoni, stipe adjacent	July 1948, Cullipool	35·9	6·7	<1·0	22·9	9·4	Nil
L. digitata, holdfast	Oct. 1948, Atlantic Bridge	32·4	8·4	<1·0	18·8	15·3	0·20
L. digitata, stipe adjacent	Oct. 1948, Atlantic Bridge	34·2	8·6	<1·0	30·6	8·1	Nil
L. saccharina, holdfast	Oct. 1948, Shuna Island	37·3	7·4	<1·0	7·5	15·9	0·18
L. saccharina, stipe adjacent	Oct. 1948 Shuna Island	34·6	7·3	<1·0	24·9	9·1	Nil
N. luetkeana, holdfast	Oct. 1948, San Juan, Wash., U.S.A.	39·0	1·2	<1·0	6·9	16·3	0·41
N. luetkeana, stipe adjacent	Oct. 1948, San Juan, Wash., U.S.A.	40·8	1·2	<1·0	13·8	5·8	0·29

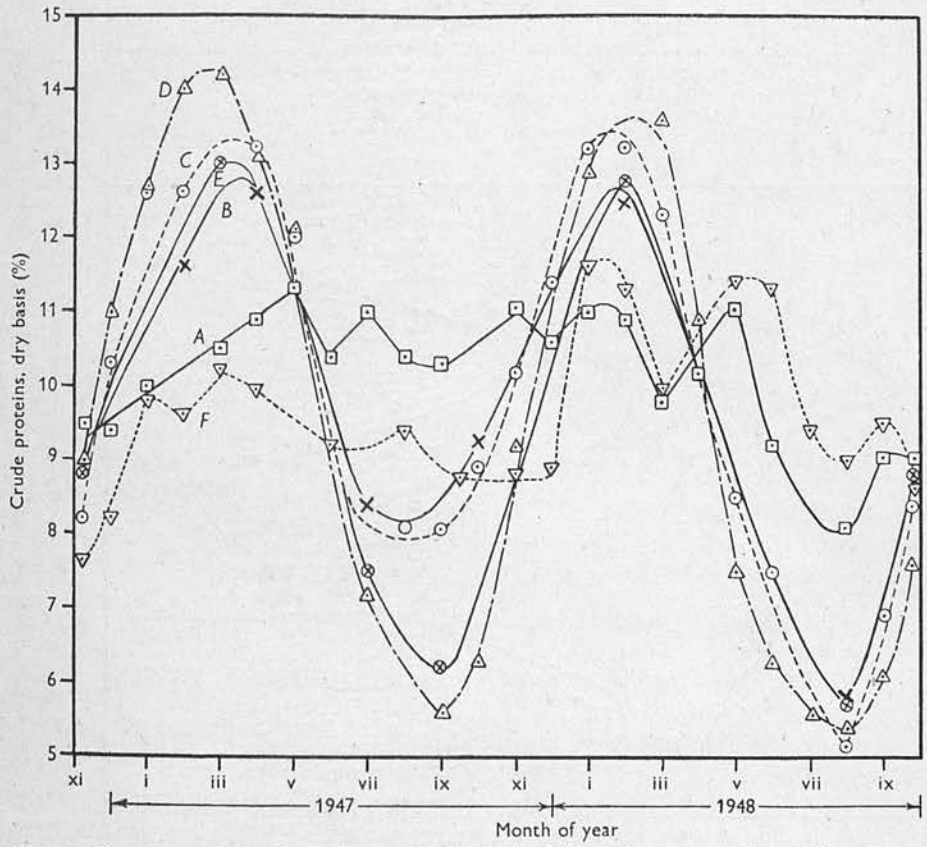

Fig. 16. Seasonal variation in crude proteins in *L. saccharina*. *A*, open-sea stipes; *B*, open-sea whole plants; *C*, open-sea fronds; *D*, loch fronds; *E*, loch whole plants; *F*, loch stipes.

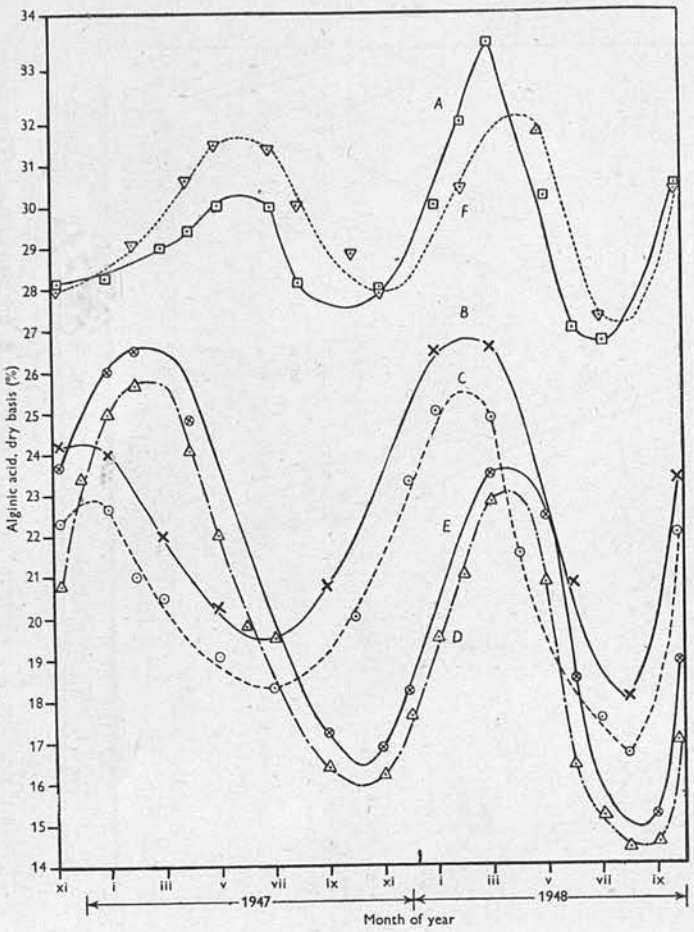

Fig. 17. Seasonal variation in alginic acid in *L. digitata*. *A*, open-sea stipes; *B*, open-sea whole plant; *C*, open-sea fronds; *D*, loch fronds; *E*, loch whole plants; *F*, loch stipes.

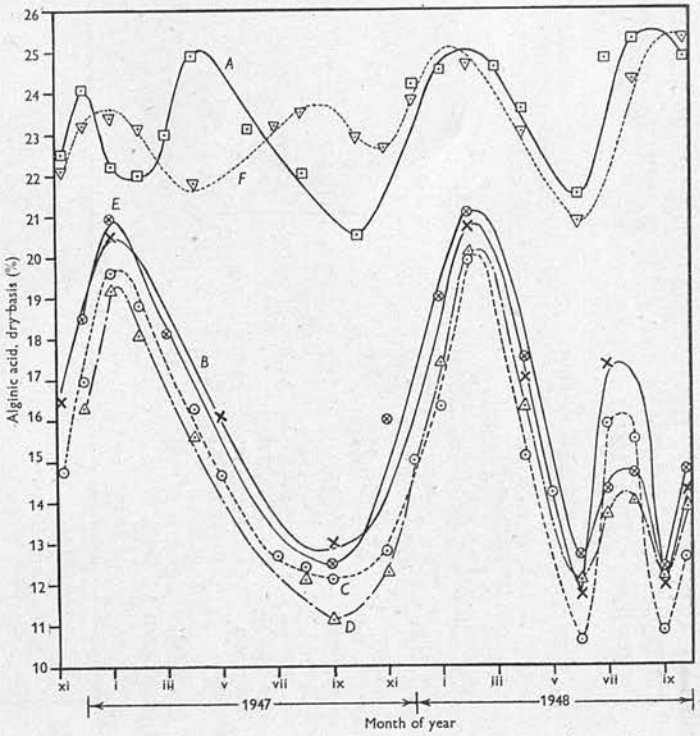

Fig. 18. Seasonal variation in alginic acid in *L. saccharina*. *A*, open-sea stipes; *B*, open-sea whole plant; *C*, open-sea fronds; *D*, loch fronds; *E*, loch whole plants; *F*, loch stipes.

outgrowths from a meristematic layer on the outside of the stipe, and once they start to form the tips can be regarded as growing tips.

It is interesting to note the low percentage of alginic acid, when determined by the standard method (Black, 1948). When the method was modified so that after soaking in acid the sodium carbonate was added without filtering off the

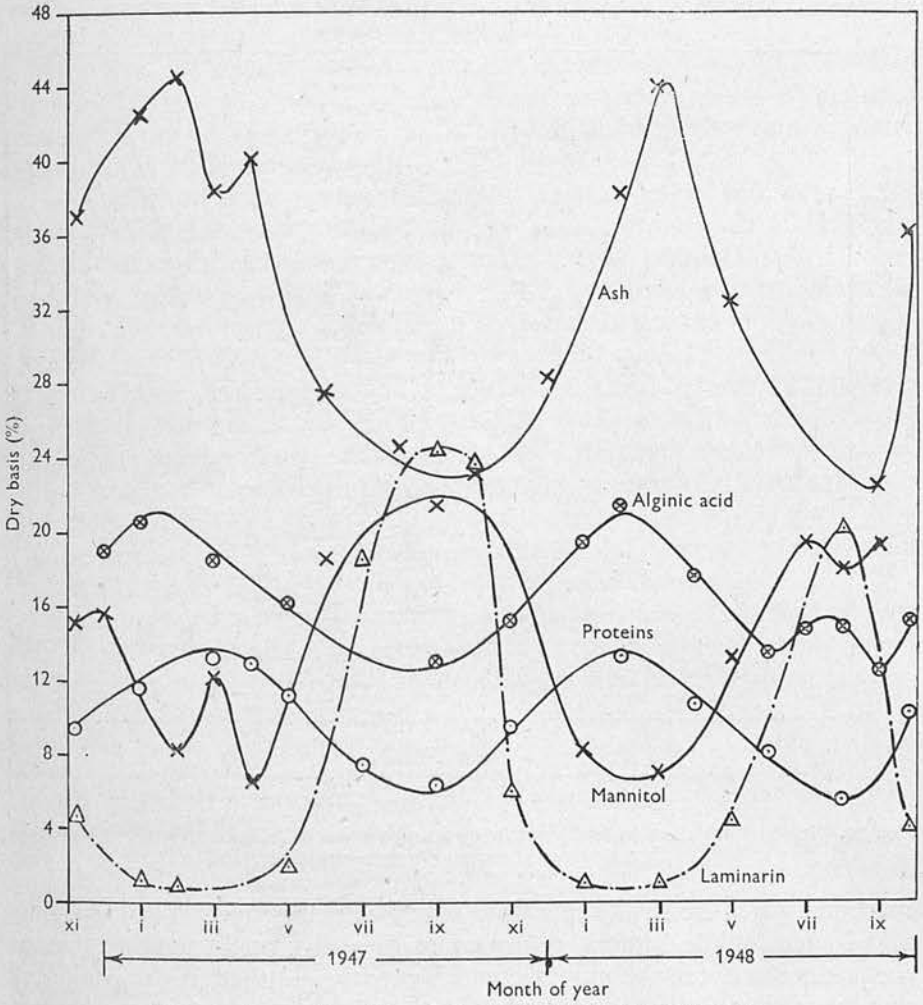

Fig. 19. Seasonal variation in *L. saccharina* (loch) whole plant.

weak acid solution, a result of 14·8% was obtained for the haptera of *Laminaria cloustoni*. When the alginic acid was determined by the carbazole method of Percival & Ross (1948*b*) a figure of 20·4% was obtained. The results indicate that in the haptera there is present either a water-soluble or a very low-grade alginate which requires further investigation.

The results show, however, that the haptera of *L. digitata* are considerably higher in alginic acid than those of *L. cloustoni* and *L. saccharina*, which may account for the very firm attachment which the holdfast of *L. digitata* has for the substratum.

The haptera as they touch the substratum become attached in a number of places. According to Parke (*loc. cit.*) they appear to secrete some sticky

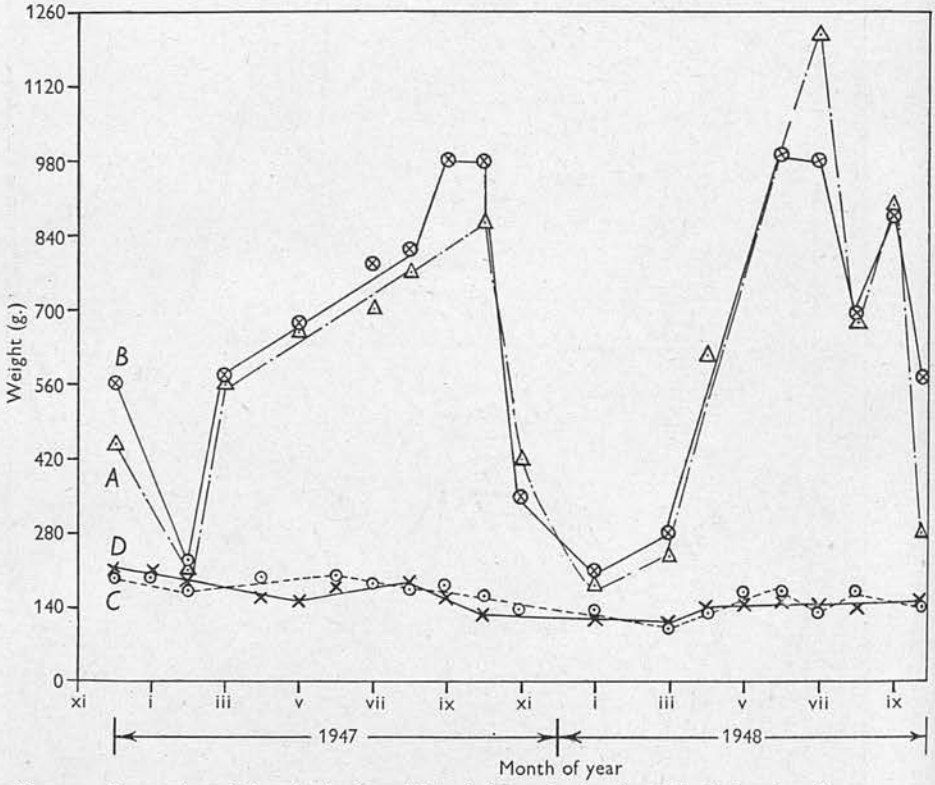

Fig. 20. Seasonal variation in fresh weight of *L. saccharina* in *A*, loch fronds; *B*, open-sea fronds; *C*, loch stipes; *D*, open-sea stipes.

substance which eventually appears as a hard chitinous layer. The low alginic acid content of the haptera, compared to the stipe, might indicate that an alginate is present in the mucilage which exudes, and there is the possibility that this alginate later becomes calcified and assists in cementing the haptera to the substratum.

Summary

The seasonal variations in the total ash, crude proteins, mannitol, laminarin and alginic acid contents are given for monthly samples of the Laminariaceae, *L. cloustoni*, *L. digitata* and *L. saccharina* from November 1946 to October

1948, samples of *L. digitata* and *L. saccharina* having been taken at different localities to determine the effect, if any, of the degree of exposure on the chemical composition.

The results agree favourably with those of the first 2 years examined and indicate that, with only a few exceptions, results might be reproducible in the corresponding season of any year, and it should be possible, therefore, to predict the approximate composition in subsequent years.

As before, the marked seasonal variations in chemical constitution occur in the fronds, where the bulk, if not all, of the photosynthesis occurs. The stipes undergo some variation parallel to that in the fronds, but within narrower limits, while laminarin is absent throughout the year.

In the fronds in the spring, mannitol is at a minimum and laminarin is absent, while the crude proteins, ash and alginic acid are at a maximum. In the autumn the reverse is true. The dry-matter content shows a corresponding variation, being at a minimum in the spring and a maximum in the autumn, but the variation is greatest in the loch samples and in *L. cloustoni* at Cullipool.

In the case of *L. digitata* and *L. saccharina*, the main effect of different degrees of exposure is that in the plants from the more sheltered localities (lochs) the laminarin and consequently the dry-weight contents are higher than in the more exposed samples, which might indicate 'restricted' growth in the lochs.

The fresh weight of the plants undergoes a similar seasonal variation, being at a minimum in the spring and a maximum in late summer.

A preliminary examination of the haptera has been carried out, and indicates a high inorganic nitrogen and free amino-acid content, while there is evidence of the presence of a lower grade alginate than is present in the adjacent stipe.

REFERENCES

ALLSOP, A., 1948. Chromatographic study of meristematic plant tissues. *Nature*, Vol. 161, pp. 833–5.

ASTBURY, W. T., 1945. Structure of alginic acid. *Nature*, Vol. 155, pp. 667–8.

ATSUKI, K. & TOMODA, Y., 1926*a*. Studies on seaweeds of Japan. First report on the chemical constitution of the Laminaria. *Journ. Soc. Chem. Ind. Japan*, Vol. 29, p. 132B.

—— 1926*b*. Chemical constituents of the brown algae. *Journ. Soc. Chem. Ind. Japan*, Vol. 29, pp. 509–17, 599.

BARRY, V. C., 1938. Preparation, properties and mode of occurrence of laminarin. *Sci. Proc. Roy. Dublin Soc.*, Vol. 21, pp. 615–22.

—— 1939. Constitution of laminarin. *Sci. Proc. Roy. Dublin Soc.*, Vol. 22, pp. 59–67.

—— 1941. Hydrolysis of laminarin. *Sci. Proc. Roy. Dublin Soc.*, Vol. 22, pp. 423–9.

—— 1942. A new method of end group assay for laminarin. *Journ. Chem. Soc.*, Vol. 183, pp. 578–81.

BARRY, V. C. & DILLON, T., 1935. Preparation and properties of alginic acid. *Sci. Proc. Roy. Dublin Soc.*, Vol. 21, pp. 165–6.

BARRY, V. C. & DILLON, T., 1936. Preparation of alginic acid and its constitution *Sci. Proc. Roy. Dublin Soc.*, Vol. 21, pp. 285–7.

BERK, F. W., 1940. Recovery of mannitol from seaweed. *Brit. Pat.*, 545,672.

BIRD, G. M. & HAAS, P., 1931. Nature of the cell wall constituents of *Laminaria* spp *Biochem. Journ.*, Vol. 25, pp. 403–11.

BLACK, W. A. P., 1948. Seasonal variation in the chemical constitution of some of th Laminariaceae common to Scotland. *Journ. Soc. Chem. Ind.*, Vol. 67, pp. 165–72

BLACK, W. A. P. & DEWAR, E. T., 1949. Correlation of some of the physical an chemical properties of the sea with the chemical constitution of the algae. *Journ Mar. Biol. Assoc.*, Vol. XXVIII, pp. 673–99.

COLIN, P. & RICARD, R., 1929. Derivatives of brown algae. Some properties c laminarin from Laminariae. *Comp. Rend. Acad. Sci. Paris*, T. 188, pp. 1449–51.

—— 1930. Glucides and derivatives of the brown algae. *Comp. Rend. Acad. Sc Paris*, T. 190, pp. 1514–16.

CORNEC, E., 1919. Spectrographic studies of the ash of marine plants. *Comp. Ren Acad. Sci. Paris*, T. 168, pp. 513–4.

DILLON, T., 1943. Utilisation of seaweed. *Chem. Age*, Vol. 49, pp. 279–83, 305–8.

DILLON, T. & MCGUINNESS, A., 1931. Alginic acid. Its mode of occurrence an constitution. *Sci. Proc. Roy. Dublin Soc.*, Vol. 20, pp. 129–33.

DILLON, T. & O'TUAMA, T., 1935. Cellulose of marine algae. *Sci. Proc. Roy. Dubl Soc.*, Vol. 21, pp. 147–52.

GOMEZ, M. L., 1933. Chemical studies of algine and alginic acid. *Int. Espan. Oceanogra Nat. Resum.*, Ser. II, No. 74, 98 pp.

GRUZEWSKA, M., 1923. Some physical and chemical properties of laminarin. *Bu Soc. Chim. Biol.*, Vol. 5, pp. 216–26.

HAAS, P. & HILL, T. G., 1929. An examination of the metabolic products of certa fucoids. *Biochem. Journ.*, Vol. 23, pp. 1001–4.

—— 1931. Preliminary note on the nitrogen metabolism of seaweeds. *Biochem. Jour* Vol. 25, pp. 1472–5.

—— 1933. Observations on the metabolism of certain seaweeds. *Ann. Bot.*, Vol. 4 pp. 55–67.

HAAS, P., HILL, T. G. & RUSSEL-WELLS, B., 1938. Observations on the metabolis of certain seaweeds. On certain simple peptides occurring in marine alg *Biochem. Journ.*, Vol. 32, pp. 2129–33.

HASSID, W. Z., 1944. The chemistry of the carbohydrates. *Ann. Rev. Biochen* Vol. 13, pp. 80–82.

HENDRICK, J., 1916. The value of seaweeds as raw materials for chemical indust *Journ. Soc. Chem. Ind.*, Vol. 35, pp. 565–74.

HIRST, E. L., JONES, J. K. N. & JONES, W. O., 1939. Structure of alginic acid. *Jou Chem. Soc.*, 1939, pp. 1880–5.

HOAGLAND, D. R. & LIEB, L. L., 1915. The complex carbohydrates and forms sulphur in marine algae of the Pacific Coast. *Journ. Biol. Chem.*, Vol. pp. 287–97.

JONES, A. J., 1922. The arsenic content of some of the marine algae. *Pharm. Jour* Vol. 109, pp. 86–7.

KIZEVETTER, I. V., 1938. Chemical composition and uses of seaweeds of far east regions. *Bull. Far East Branch Acad. Sci. U.S.S.R.*, No. 31, pp. 49–109.

KREFTING, A. & TORUP, R., 1909. A new carbohydrate from the Laminariace *Pharmacia*, Vol. 6, pp. 151–3.

KYLIN, H., 1913. Biochemistry of sea algae. *Ztschr. Physiol. Chem.*, Bd. 83, pp. 171–

—— 1915. Biochemistry of sea algae. *Ztschr. Physiol. Chem.*, Bd. 94, pp. 337–42

KYLIN, H., 1918. Chemistry of seaweeds. *Ztschr. Physiol. Chem.*, Bd. 101, pp. 236–47.
—— 1944. The biochemistry of the Phaeophyceae. *Kgl. Fysiograf. Sällskap. Lund, Förh.*, Bd. 14, pp. 226–38.
LAPICQUE, L., 1919. Seasonal variations in the chemical composition of the marine algae. *Comp. Rend. Acad. Sci. Paris*, T. 169, pp. 1426–8.
LE GLOAHEC, V. C. E. & HERTER, J. R., 1940. Removal and recovery of laminarin. *U.S. Patent*, 2,188,092.
LUNDE, G., 1937. Seaweed as source of chemical raw materials. *Ztschr. Angewandte Chemi.*, Bd. 50, pp. 731–4.
MANNING, W. M. & HARDIN, G., 1944. Zanthophylls and carotenes in brown algae. *Biol. Bull. Wood's Hole*, Vol. 86, pp. 169–91.
NAYLOR, G. C. & RUSSEL-WELLS, B., 1934. Presence of cellulose and its distribution in the cell walls of brown and red algae. *Ann. Bot.*, Vol. 48, pp. 635–41.
NELSON, W. L. & CRETCHER, L. H., 1929. The alginic acid from *Macrocystis pyrifera*. *Journ. Amer. Chem. Soc.*, Vol. 51, pp. 1914–22.
NISIZAWA, K., 1940. Laminarin and mannitol of brown algae. *Science Repts. Tokyo Bunrika Daigaku*, Vol. 5B, pp. 9–14.
ÖY, E., 1940. Content of iron, copper, manganese and boron in seaweeds. *Tids. Kemi Bergv.*, Vol. 20, pp. 114–17.
PARKE, M., 1948. Studies on British Laminariaceae. I. Growth in *Laminaria saccharina*. *Journ. Mar. Biol. Assoc.*, Vol. 27, pp. 651–707.
PERCIVAL, E. G. V. & ROSS, A. G., 1948*a*. The cellulose of marine algae. *Nature*, Vol. 162, pp. 895–7.
—— 1948*b*. Colorimetric method for estimation of alginic acid. *Journ. Soc. Chem. Ind.*, Vol. 67, pp. 420–1.
RICARD, R., 1931. Nature and seasonal variation of the carbohydrates in laminaria. *Bull. Soc. Chim. Biol.*, Vol. 13, pp. 417–35.
RUSSEL-WELLS, B., 1932. Fats of brown seaweeds. *Nature*, Vol. 129, pp. 654–5.
SCHMIEDEBERG, W. 1885. Über die Bestandtheile der Laminaria. *Gesellschaft deutscher Naturforscher und Arzte, Leipzig*, Tageblatt der Versammlung, No. 58, p. 427.
SPEAKMAN, J. B. & CHAMBERLAIN, N. H., 1944. Structure of alginic acid. *Journ. Soc. Dyers and Col.*, Vol. 60, pp. 264–72.
STANFORD, E. C. C., 1883. On algin: a new substance obtained from some of the common species of marine algae. *Chem. News*, Vol. 47, pp. 254–7, 267–9.
—— 1884*a*. On algin. *Journ. Soc. Chem. Ind.*, Vol. 3, pp. 297–302.
—— 1884*b*. The economic applications of seaweed. *Journ. Soc. Arts, London*, Vol. 32, pp. 717–30.
—— 1886. On alginic acid and its compounds. *Journ. Soc. Chem. Ind.*, Vol. 5, pp. 218–21.
STENHOUSE, J., 1844. On the occurrence of mannite in *L. saccharina* and other seaweeds. *Journ. Chem. Soc.*, Vol. 2, pp. 136–40.
STEWART, W. T. & LUCAS, H. T., 1940. Oxidation of alginic acid by periodic acid. *Journ. Amer. Chem. Soc.*, Vol. 62, pp. 1792–6.
TAKAHASHI, E., SIRAHAMA, K. & SHUN-ITI-TASE, 1933. Chemical studies on fats of algae. Part I. Contents of fats and their properties. *Journ. Chem. Soc. Japan*, Vol. 54, pp. 619–23.
—— 1935. Part II. Contents of fats and their properties. *Journ. Chem. Soc. Japan*, Vol. 56, p. 1250.
—— 1939. Part V. Non-volatile fatty acids of *Alaria crassifolia*. *Journ. Chem. Soc. Japan*, Vol. 60, pp. 56–60.

Reprinted from the Journal of the Marine Biological Association of the United Kingdom, Vol. xxviii, December 1949, pages 673–699

PRINTED IN GREAT BRITAIN

CORRELATION OF SOME OF THE PHYSICAL AND CHEMICAL PROPERTIES OF THE SEA WITH THE CHEMICAL CONSTITUTION OF THE ALGAE

By W. A. P. Black, B.Sc., Ph.D., F.R.I.C.
and E. T. Dewar, B.Sc., Ph.D.
Scottish Seaweed Research Association, Musselburgh

(Text-figs. 1–14)

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PART I. THE SEASONAL VARIATION IN TEMPERATURE, pH, SALINITY, DISSOLVED OXYGEN, PHOSPHATE, AND NITRATE IN THE INSHORE WATERS AT THREE POSITIONS ON THE ARGYLLSHIRE COAST

INTRODUCTION

Although reliable data are available for the seasonal variation of the nutrient salts of surface off-shore waters in various parts of the world (Harvey, 1945, pp. 75–8, 85–6), information on inshore coastal waters is scanty. In North

America, however, extensive investigations have been carried out within the last 20 years on the coastal waters bordering on the states of Washington and British Columbia. These waters are cut off from the Pacific Ocean by Vancouver Island, and thereby possess certain characteristic properties. Hutchinson, Lucas & McPhail (1929) have described the seasonal variations in the waters of the Strait of Georgia. The phosphate figures are much higher than in the open sea, and these authors attribute the high nutrient content to the inflow of water from the Fraser River.

The waters of Puget Sound are also particularly rich in phosphates, nitrates and silicates. Thompson & Robinson (1933) believe this to be due to upwelling off the entrance to Juan de Fuca Strait and to vigorous mixing and agitation of the waters. Phifer & Thompson (1937) give the results of 5 years' study of the inshore conditions at Friday Harbour, on the San Juan Channel. In general, the nitrate and phosphate figures are about four times higher than those reported in our waters, and they are not subject to the same marked seasonal variation, while dissolved oxygen saturations rarely exceed 80%. In this channel strong tidal currents throughout the year create turbulent conditions, which are responsible for the high nutrient and low oxygen contents of these waters.

Newcombe, Horne & Shepherd (1939) have studied the waters of Chesapeake Bay, in the region of Solomons Island, Maryland, U.S.A., during the period 1936–38. Conditions in Chesapeake Bay, however, are typically 'estuarine', surface chlorinities being low (5·8–10·2‰) and summer temperatures high (25° C.). The phosphate content of the Bay waters in summer commonly ranges about $0{\cdot}23 \times 10^{-3}$ mg. atom P/l. at the surface, while the river waters may contain $1{\cdot}0 \times 10^{-3}$ mg. atom P/l. Minimum concentrations are obtained during the winter period, when the abundance of phytoplankton organisms is maximum. Estuarine waters are rich in ammonium and silicate.

Marshall & Orr (1927) obtained a close relation between the growth of diatoms and chemical changes in the Clyde Sea Area in 1924, 1925 and 1926 and again in 1927 and 1928 (1930). In 1926 the seasonal variation in temperature, density, pH, salinity, dissolved oxygen and phosphate were determined for two points in Loch Striven. The two localities visited were Clapochlar (depth *c.* 60 m.) and Loch Striven head (depth *c.* 28 m.), and samples were taken at 0, 10, 20, 40 and 60 m. at weekly intervals from the end of January till the end of November. Loch Striven is not polluted to any extent by the Clyde, although the surface water is often affected by drainage from the hills, which give rise to erratic salinity values.

No other work, at least to the writers' knowledge, appears to have been carried out on the seasonal variation in the nutrient content of the Scottish inshore waters, and it seemed possible that lack of nutrients during the summer months might influence the growth of algae.

This investigation was undertaken, therefore, in order to determine the

seasonal variation in the nutrient content of our sea water and to ascertain if any correlation existed between the composition of the sea water, in particular, nitrate, and that of the algae growing in it.

Location and Methods of Sampling

The three positions selected were Eilean Coltair (at the entrance to Loch Melfort), Rudh-an-Aoil (Shuna Island) and Cullipool (Luing Island). Eilean Coltair is the most sheltered of the three positions, being situated in a loch with the water cut off to a certain extent. Shuna Island is more exposed, with strong tidal currents running, but it is protected from the open sea by the Island of Luing. Cullipool, situated on the north-west coast of Luing Island, is open to the Atlantic and is the most exposed position.

The upper waters pass in general direction from the Irish Sea, through the North Channel and up the west coast of Scotland, through the Minch and round the north coast to the Orkney region. The water at the three positions selected can therefore be regarded as typical coastal inshore water, practically free from oceanic water.

Samples from the three localities were taken monthly for 13 months from March 1948 to March 1949 by means of a Nansen-Pettersson water bottle at a depth of 4–5 m. (low water), just above the weed bed. Temperatures were recorded with a reversing thermometer and the transparency of the waters measured with a standard Secchi disk.

The samples of water, carefully protected from light, were transported to Inveresk Gate and the chemical analysis carried out within 24 hr.

Experiments and Results

The usual methods for the various determinations were utilized, and the results are summarized in Tables I and II and Figs. 1–6.

The pH was measured with the Marconi pH meter, and the observed readings corrected for the rise in temperature since sampling. The pH values for the three localities are given in Table I but, because of the relative constancy for any given month during the period, only the Shuna Island figures are reproduced in Fig. 5. Dissolved oxygen was estimated only in the Shuna Island waters and the results are given in Table II and Fig. 3.

Nitrate was determined by the diphenylbenzidine method of Atkins (1932), which was modified slightly, as outlined below, to allow the colour intensities to be measured by the Spekker Photoelectric Absorptiometer. The intensity of the blue colour which develops does not bear a linear relationship to the nitrate content, but the graph of colour intensity plotted against nitrate concentration gives a smooth curve. The slope of this curve increases with increased nitrate content, so that the higher nitrate figures are more accurate than the lower figures. The accuracy of the method, however, increases from

TABLE I. SEASONAL VARIATION IN TEMPERATURE, pH AND TRANSPARENCY

Month	Temperature (°C.)			pH *in situ*			Secchi disk (m.)		
	Eilean Coltair	Shuna Island	Culli-pool	Eilean Coltair	Shuna Island	Culli-pool	Eilean Coltair	Shuna Island	Culli-pool
1948									
March	7·5	7·5	—	8·03	7·96	—	—	—	—
April	8·2	8·1	7·8	8·09	8·11	8·11	9	9	5
May	9·8	9·8	9·0	8·17	8·13	8·10	7	7	9
June	12·4	11·1	10·6	8·13	8·13	8·10	7	8	9
July	12·2	12·3	11·7	8·11	8·12	8·11	9	7	5
August	13·0	13·0	12·7	7·91	7·95	7·96	9	9	7
September	12·5	11·9	12·2	7·86	7·91	7·90	7	9	7
October	12·2	11·9	12·1	8·01	8·06	8·06	7	6	5
November	10·5	10·9	11·0	8·01	8·04	8·06	5	7	7
December	9·7	10·0	10·2	7·96	8·02	8·05	8	7	7
1949									
January	6·5	7·0	8·1	8·04	8·07	8·07	7	7	7
February	7·8	7·7	8·0	8·01	8·03	8·05	7	7	5
March	6·1	6·9	6·9	8·04	8·05	8·07	7	7	7

TABLE II. SEASONAL VARIATION IN SALINITY, OXYGEN, PHOSPHATE AND NITRATE

Month	Salinity (g./kg.)			Oxygen. Shuna Island		Phosphate (mg. atoms P/m.³)			Nitrate (mg. atoms N/m.³)		
	Eilean Coltair	Shuna Island	Culli-pool	ml./l. at N.T.P.	% saturation	Eilean Coltair	Shuna Island	Culli-pool	Eilean Coltair	Shuna Island	Culli-pool
1948											
March	34·05	34·12	—	—	—	0·55	0·55	—	6·4	6·1	—
April	33·56	33·81	33·94	6·24	93·5	0·35	0·52	0·41	0·7	3·1	5·0
May	33·78	33·86	34·02	6·14	95·4	0·19	0·26	0·33	1·0	2·6	4·6
June	33·90	33·93	33·93	5·94	94·9	0·16	0·20	0·18	1·4	2·4	1·6
July	33·83	33·72	33·92	6·02	98·2	0·16	0·16	0·20	0	0	0·9
August	34·01	34·02	34·05	5·73	94·9	0·19	0·19	0·20	0·5	1·0	1·5
September	33·61	33·06	33·76	5·83	93·9	0·30	0·23	0·30	3·0	3·1	4·9
October	33·63	33·57	33·65	5·71	92·4	0·37	0·31	0·31	5·0	5·4	4·8
November	33·34	33·67	33·83	5·86	93·0	0·40	0·50	0·42	4·9	5·6	5·7
December	33·16	33·59	33·83	5·85	90·4	0·75	0·65	0·50	5·5	6·1	5·7
1949											
January	32·92	33·26	33·44	6·41	92·8	0·57	0·53	0·53	6·3	6·5	6·9
February	33·45	33·62	33·56	6·40	94·3	0·51	0·53	0·53	6·5	6·7	6·5
March	33·62	33·83	34·03	7·00	101·5	0·48	0·53	0·52	5·5	5·8	5·9

± 10 mg. at 0–30 mg. nitrate – N/m.³ to ± 4 mg. at 90 mg. N/m.³. This method cannot compete with Harvey's strychnidine method at low nitrate concentrations, for the latter gives a linear relation between colour intensity and nitrate content. It was not used, however, in these investigations, because of the difficulty of preparing a satisfactory reagent.

Fig. 1. Seasonal variation in temperature. (*A*) L. Melfort; (*B*) Shuna Island; (*C*) Cullipool.

Fig. 2. Seasonal variation in phosphate. (*A*) L. Melfort; (*B*) Shuna Island; (*C*) Cullipool.

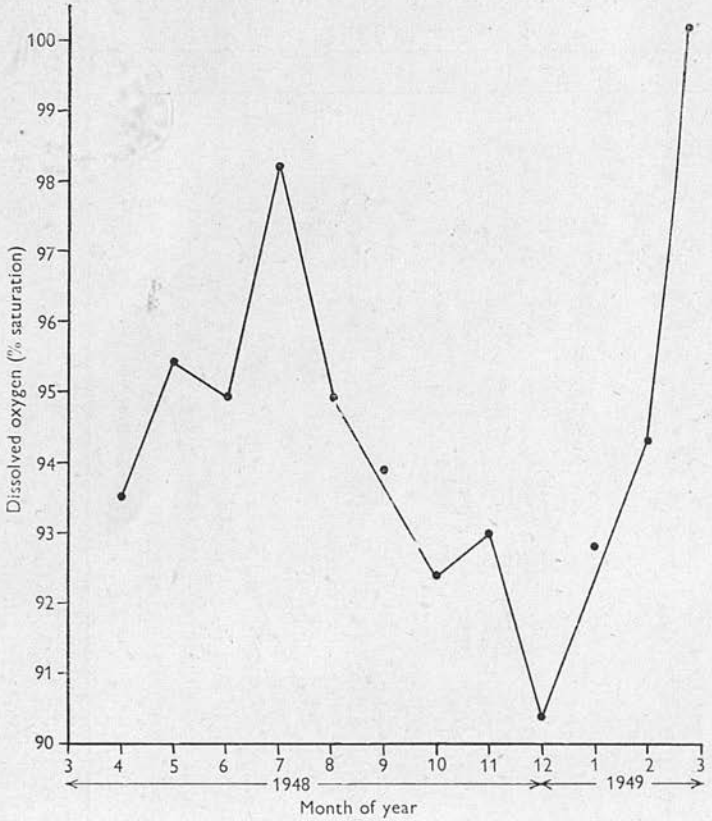

Fig. 3. Seasonal variation in dissolved oxygen. Shuna Island.

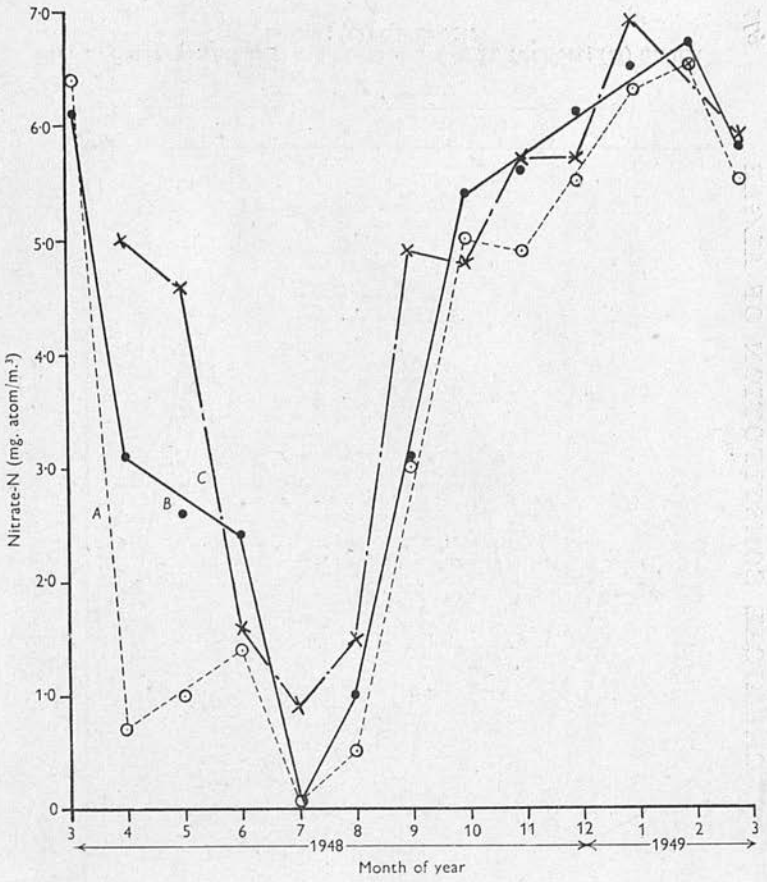

Fig. 4. Seasonal variation in nitrate. (*A*) L. Melfort; (*B*) Shuna Island; (*C*) Cullipool.

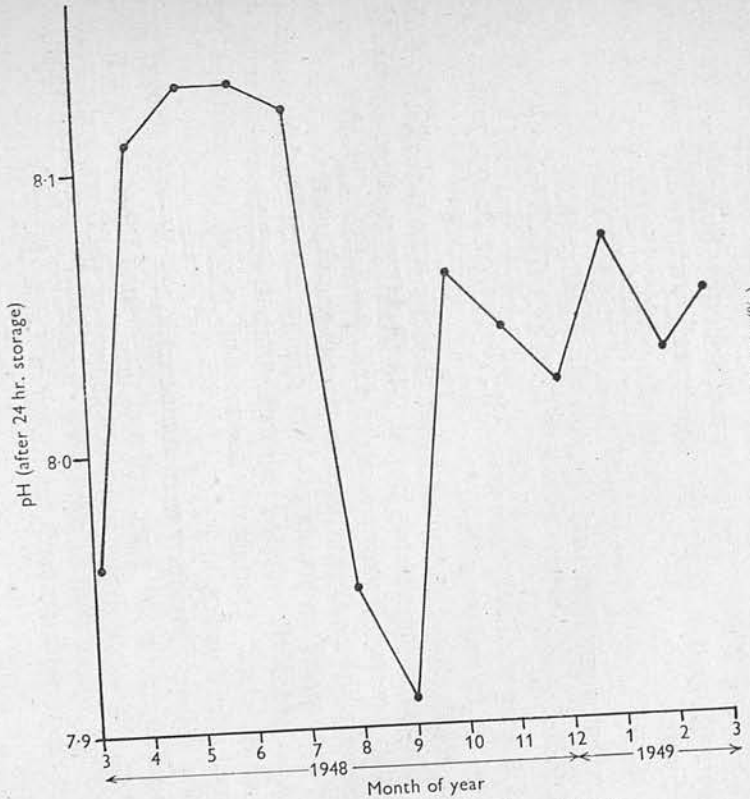

Fig. 5. Seasonal variation in pH. Shuna Island.

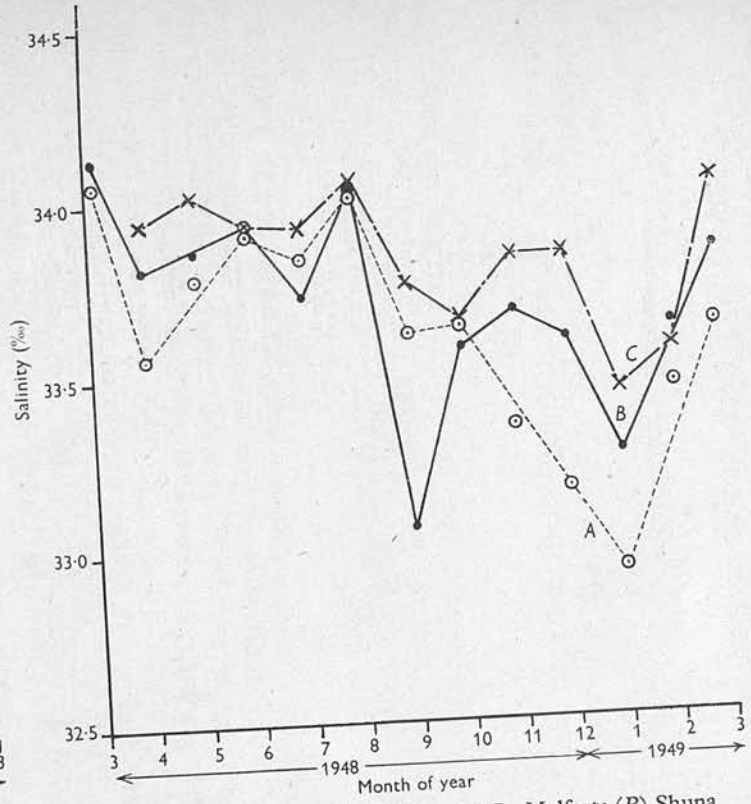

Fig. 6. Seasonal variation in salinity. (*A*) L. Melfort; (*B*) Shuna Island; (*C*) Cullipool.

Estimation of Nitrate

REAGENTS

Sulphuric Acid. 1 l. of H_2SO_4 (J. F. MacFarlan and Co's special nitrate free acid) was freed from traces of nitrate by heating at 300° for 2 hr. with $(NH_4)_2CO_3$ (3 g.).

Diphenylbenzidine Reagent. The diphenylbenzidine was recrystallized from boiling toluene. 20·0 mg. were dissolved in 100 ml. H_2SO_4, the solution thoroughly shaken and allowed to stand several days before use. The final reagent was faintly blue.

Standard Nitrate Solutions. Stock Solution A: 0·7218 g. A.R. KNO_3, made up to 1 l. with distilled water, gives a solution containing 0·1 g. N/litre. *Solution B:* 10 ml. solution *A* were diluted to 1 l. with distilled water or nitrate-free sea water, as required, to give a solution containing 1 mg. N/l, i.e. 1000 mg. N/m.^{3}. *Standards C:* These were prepared from solution *B* by suitable dilution with distilled water or nitrate-free sea water as required. Solution *B* and all standards must be freshly prepared each day during calibration of the reagent.

Nitrate-free Sea Water. The sea water collected from Eilean Coltair on 11 May 1948 gave a blank not significantly greater than that given by distilled water.

CALIBRATION OF THE REAGENT

(1) *In Distilled Water*

The following standards were prepared: 100, 95, 90, 80, 75, 70, 60, 55, 50, 45, 40, 35, 25, 10 and 0 mg. N/m.^{3}.

2·5 ml. standard in a stoppered weighing bottle were treated with 6·0 ml. H_2SO_4, mixed and immediately cooled. The H_2SO_4 was added by means of a graduated pipette, allowing exactly 1 min. for delivery in each case. A stoppered weighing bottle was used to prevent the solution taking up water from the atmosphere on standing. After cooling, 1·5 ml. diphenylbenzidine reagent were added, the solution thoroughly mixed, and allowed to stand for 22·5 hr. in the dark. During the development period, the solutions were again mixed after 6 and 22 hr.

The blue colours developed were compared in the Spekker Photoelectric Absorptiometer, using the 1 cm. cell and the orange Ilford 607 filter. For convenience, the water setting was taken as 0·70 on the drum scale, and the drum readings found (log I) subtracted from 0·70 (log I_0). The values of log I_0/I for the various standards are shown in Table III.

TABLE III

Distilled water		Distilled water		Sea water		50% (v/v) sea water	
Standard	log I_0/I	Standard	log I_0/I	Standard	log I_0/I	Standard	log I_0/I
100	0·523	50	0·203	100	0·331	100	0·468
95	0·485	45	0·177	75	0·211	75	0·295
90	0·456	40	0·162	50	0·136	50	0·161
80	0·413	35	0·141	25	0·080	25	0·088
75	0·329	25	0·080	10	0·050	10	0·048
70	0·324	10	0·042	0	0·035	0	0·032
60	0·238	0	0·030				
55	0·239						

There is not a linear relationship between colour intensity (log I_0/I) and nitrate concentration, but the graph gives a smooth curve.

(2) *In Sea Water*

In order to determine the salt error, a calibration curve was carried out using nitrate-free sea water in the preparation of the standards. The results, shown in Table III, indicate that the salts in sea water decrease the colour formation, when the colour is measured after 22·5 hr.

(3) *In* 50% *(v/v) Sea Water*

Solution *B* was made up in distilled water. The standards were prepared by taking the appropriate amount of *B*, adding 50 ml. nitrate-free sea water and making up to 100 ml. with distilled water. The results in Table III show that the colour intensities lie, for the most part, between the distilled water and sea-water values.

ESTIMATION OF NITRATE IN SEA-WATER SAMPLES

2·5 ml. of the unknown sample were treated with H_2SO_4 and diphenylbenzidine exactly as described in the calibration of the reagent, and the value of log I_0/I determined after 22·5 hr. The nitrate content of the sample in mg. nitrate-N/m.^{3} was read off from the sea-water calibration curve.

ACCURACY OF THE METHOD

It was found that the value of log I_0/I for any given standard could, in general, be reproduced within $\pm 0{\cdot}02$. From the sea-water calibration curve, the accuracy at various concentrations has been estimated as follows:

Nitrate concentration	Maximum error
30 mg. N/m.^{3}	$\pm$10 mg. N/m.^{3}
40	$\pm 8{\cdot}5$
50	$\pm 7{\cdot}5$
60	$\pm 7{\cdot}0$
70	$\pm 5{\cdot}5$
80	$\pm 4{\cdot}5$
90	$\pm 4{\cdot}0$

Estimation of Phosphate

Phosphate was estimated by the Denigés method, as described by Wattenberg (1937).

Discussion of Results

Temperature

In the spring, temperatures are at a minimum, 6–7° C. (Table I and Fig. 1). The loch water at Eilean Coltair gets warmer more rapidly than the open sea, reaching a temperature of over 12° C. by June. At Cullipool the surface waters take considerably longer to heat up, 12° C. only being reached in August. For Shuna Island, in general, the graph lies between the other two.

The opposite state of affairs prevails during the autumn and winter months. Cullipool maintains a temperature of 8° C. or more until February, while the loch water has dropped to 6·5° C. by January. One would expect loch water, because of its proximity to, and isolation by, land, to warm up and cool down more rapidly than the open sea. It is interesting to note that the maximum and minimum temperatures for the three positions do not vary appreciably, although the times required to reach these values do.

Marshall & Orr (1927) found the temperature of the surface waters (0–10 m.) at Clapochlar to be at a minimum (6° C.) in January/February, rising to a maximum of 14° C. in August.

pH

The pH was measured 24 hr. after sampling. The effect of storage is to lower the pH for the following reasons: (1) On standing, the water warms up to that of its surroundings, and the pH falls with rising temperature. The pH *in situ*, however, can be calculated from the pH at temperature of observation by allowing for the temperature coefficient of sea water. In most cases, the decrease in pH per 1° C. rise can be taken as 0·01 pH unit. (2) Bacteria liberate carbon dioxide in respiration, and this lowers the pH. This effect cannot be corrected for, and may be appreciable during warm weather. Although care was taken to keep the temperature of the samples as low as possible during transport, the results may be slightly low.

In April a rapid increase in pH occurs, indicating that plant growth is beginning to utilize carbon dioxide at a greater rate. From April to July the pH values for all positions are 8·10 or greater, but in August and September figures of 7·86–7·96 indicate a slowing up in the rate of photosynthesis which coincides with a low nutrient content of the water. An increase in pH occurs in October, and from October to March fairly constant values of $8{\cdot}00 \pm 0{\cdot}07$ are obtained.

The pH of the surface water at Clapochlar (Marshall & Orr, 1927) was close to 8·0 during the early part of the year. This was followed by a sudden increase at the beginning of April following the spring diatom outburst, and figures of 8·3–8·4 are recorded during this period. Another peak in the pH graph was obtained in September due to the autumn phytoplankton growth.

Transparency

It was hoped, by means of a Secchi disk, to detect the phytoplankton outburst in the spring, but in practice very little variation was observed throughout the year. Maxima of 7–9 m. and minima of 5–6 m. are recorded, but these do not occur at any specific time and appear to have no significance.

Salinity

In ocean water away from the influence of land, variations in surface salinity are not great. Böhnecke (1938) has shown for an area of the North Atlantic Ocean, extending between latitudes 18° and 42° N., that the highest average surface salinity, 36·70‰, occurs in March and the lowest, 36·59‰, in November. The variations from one month to another are irregular, but on the whole the salinity is somewhat higher in spring than it is in autumn. In the open ocean variations in surface salinity depend mainly upon variations in the difference between evaporation and precipitation, in association also with currents. In inshore waters salinity values are complicated by the influence of land, which gives rise to coastal currents and dilution due to rivers and land drainage. For example, when heavy rain falls during the winter the increased volume of waters discharged by the rivers of the region will tend to decrease the salinities during the winter months. If, however, the precipitation is in the form of snow, the effect upon the salinity of the coastal waters may not be experienced until the summer.

The high salinity values obtained in this investigation, particularly for Cullipool, indicate that these waters have not been diluted by fresh water to any great extent, and the figures bear some relation to the rainfall for the 13 months. For all positions, highest values were obtained in March and August, and lowest values in January. In September an abnormally low figure, 33·06‰, was reported for Shuna Island. In general, the salinity of Cullipool water is higher than the loch water, and this is to be expected because land drainage after heavy rain will affect loch more than open sea water. Again, the graph for Shuna Island lies between the other two for most months of the year, although there are some exceptions.

Marshall & Orr (1927) found the salinity of the surface layer at Clapochlar lay between 33 and 34‰, although some values for the immediate surface layer were much lower, for example, some very low figures are recorded during March and October/November.

Oxygen

With the exception of March 1949, the samples were treated with the reagents, manganous chloride and sodium hydroxide-potassium iodide, about 24 hr. after sampling. In March 1949, the reagents were added immediately after sampling, and the iodine liberated and titrated the following day. This water was found to be slightly supersaturated (% saturation, 101·5). Thus it may be

assumed that all the results, except the last, are low to the extent of 5–10%. Nevertheless, the relative values are of interest in detecting the period of photosynthesis in the spring. The explanation for these low results is twofold: (1) on standing, the water warms up to that of its surroundings, and a certain amount of oxygen tends to be expelled from solution at the higher temperature; and (2) when sea water is stored, bacteria rapidly consume oxygen in respiration, the amount utilized being quite appreciable even after 24 hr. Moreover, bacteria are particularly abundant in inshore water.

The highest saturations are obtained during the spring and summer months, the maximum (98·2%) being reached in July, which is further evidence of photosynthesis during these months. Saturations then decrease steadily to a minimum of 90·4% in December. Because of the uncertainty of these results, further discussion would be unwise, but it appears that this water is saturated, or very nearly saturated, for the greater part of the year, and probably slightly supersaturated during the period of photosynthesis.

Marshall & Orr (1927) found the dissolved oxygen saturations for the surface layers were close to 100% throughout the year, except during the diatom outbursts in the spring and autumn when values up to 138% were occasionally recorded.

Phosphate

The phosphate estimations were carried out one day after sampling, the water being centrifuged before analysis if any turbidity was noticed. It is advisable, however, to estimate phosphate immediately after sampling, or to saturate the water with chloroform if estimations are to be carried out at a later date. On standing, the inorganic phosphate may increase slightly if much organic phosphorus is present, or it may decrease due to utilization by bacteria, but this error will not be great after only 24 hr., provided the temperature of the water is prevented from rising appreciably.

Fig. 2 indicates that the phosphate content begins to decrease after March, the decrease being most marked in the loch water, although the open sea at Cullipool has a phosphate content in April not much greater than the loch. Minimum values of 0·16–0·20 mg. atom phosphate-P/m.^{3} are obtained in all localities by June, and remain at this low level until September, when there is an increase again to the winter maximum in December, January and February. Abnormally high values were obtained in December for Eilean Coltair (0·75 mg. atom P/m.^{3}) and Shuna Island (0·65 mg. atom P/m.^{3}), which can only be attributed to contamination by land drainage, as the value for Cullipool was normal.

The winter maximum for 1949 of 0·52 mg. atom P/m.^{3} is in good agreement with the values quoted for the English Channel, although the summer minimum tends to be higher than that found in the Channel.

The seasonal variation in phosphate at a position in the English Channel

has been followed at monthly intervals for a number of years. From winter maximum values varying between 0·69 and 0·47 mg. atom P/m.^{3} it has fallen in some years to less than 0·02 in the surface layers, in other years 0·06 or 0·10 mg. atom remain (Atkins, 1923–30; Cooper, 1933*a*, *b*, 1938). The winter maximum values of phosphate in the water at the beginning of the year are not constant but fluctuate from year to year. Cooper (1938) has found lower winter maxima during the 1930's as compared with the 1920's, and these have been reflected in decreased plankton and fish populations (Russell, 1935, 1936*a*, *b*).

During February at Clapochlar (Marshall & Orr, 1927), phosphate in the surface layer was about 0·65 mg. atom P/m.^{3}. Within a few weeks, at the end of March and beginning of April, phosphate was reduced to 0·16 mg. atom P/m.^{3}, while by the end of May no trace of phosphate existed in the surface layer. Small quantities appeared in the waters at odd periods throughout the summer, but no full-scale regeneration of phosphate became apparent until the end of September. Maximum winter values were obtained by November.

Nitrate

The estimations were carried out 1 day after sampling. The effect on the nitrate content of storing samples for 24 hr. is negligible, provided the samples are shielded from light. Fig. 4 shows that the seasonal variation of nitrate follows closely that of phosphate. After March nitrate begins to decrease, and again this decrease is most marked in the loch. Between March and April 1948 nitrate-N in Loch Melfort dropped from 6·4 mg. atoms/m.^{3} to almost zero, and remained at this level until September. Complete exhaustion of nitrate was not observed at Shuna Island and Cullipool until July. Nitrate is almost certainly absent in all localities during July and August. As in the case of phosphate, nitrate is regenerated during September and increases gradually during the autumn and winter, reaching a maximum of about 6·8 mg. atoms N/m.^{3} in January. Little or no evidence was obtained of the plankton outburst in the autumn, either from the phosphate or nitrate records.

The seasonal variation in nitrate has been followed during the course of several years in the English Channel (Harvey, 1926, 1928; Cooper, 1933*a*). Exhaustion of nitrate due to utilization by phytoplankton is frequently found in the surface water during the summer, while winter maxima may exceed 7 mg. atoms nitrate-N/m.^{3}. The absence of nitrate, however, does not necessarily mean that plant production in the sea is reduced to a low level, as nitrogen may still be available as ammonium, the concentration of which may reach 2 mg. atoms ammonium-N/m.^{3} during the autumn and winter months. Harvey (1940) has shown that plankton diatoms utilize ammonium in preference to nitrate. This may explain why the autumn plankton outburst was not detected by following the seasonal variation in nitrate (Fig. 4), although the flattening of the graph from September to December might be taken as an

indication of its utilization by plankton. In the spring, nearly all the available nitrogen is present as nitrate.

The Nitrogen/Phosphorus Ratio at the Winter Maximum

If 0·52 mg. atom P/m.^{3} and 6·8 mg. atoms N/m.^{3} are taken as the winter maximum values for phosphate and nitrate respectively in 1949, the N/P ratio is 13·2.

The significance of these results on the growth of the adjacent seaweeds is discussed in Part II.

Summary

Monthly samples of sea water, taken from three localities on the Argyllshire coast, from March 1948 to March 1949, have been analysed for pH, dissolved oxygen, salinity, nitrate and phosphate, while temperatures and transparencies have been recorded.

Surface temperatures range from 6–7° C. in January/March to 13° in August. Transparencies remain fairly constant at 7–9 m. throughout the whole period, with occasional minimum values of 5–6 m.

pH values close to 8·0 are obtained from October to March, followed by an increase to 8·1 or over during the period of photosynthesis (April to July). Low pH values (7·86–7·96) are recorded during August and September.

Salinities vary over the narrow range, 33–34 °/$_{00}$, with maxima in March and August and minima in January. These high salinities indicate that the inflow of fresh water from streams in the neighbourhood is negligible, even for Loch Melfort.

Dissolved oxygen saturations are highest in the spring and summer months and lowest in December.

Phosphate and nitrate begin to decrease after March, the reduction being most marked in the loch, and remain at a low level until September, when these nutrients are again regenerated. Winter maximum values of 0·52 mg. atom P/m.^{3} and 6·8 mg. atoms nitrate-N/m.^{3} are obtained by January.

Atkins's diphenylbenzidine method has been used to estimate nitrate, and a description of the method is given.

PART II. CORRELATION OF THE COMPOSITION OF SEA WATER WITH THE CHEMICAL CONSTITUTION OF THE ALGAE

INTRODUCTION

The larger algae, like most other forms of marine plant life are, apart from the influence of light, wholly dependent for their growth and development on the nutrient content of the surrounding sea water.

Although there is no experimental evidence to show that the growth rate of sea-weeds in the sea is ever brought to a standstill through lack of nutrients, Harvey's work (1926) indicated that lack of nitrate can limit plant growth. He also showed (1933) that a marked reduction in the rate of photosynthesis for the diatom *Nitzschia closterium* occurred with phosphate concentrations below 0·32 mg. atom P/m.^{3}, being most marked below 0·16 mg. atom P/m.^{3}. There appears to be no doubt that nitrate and phosphate are most important factors in the productivity of the sea, and plankton, at least, is richer where vertical currents aid in increasing their concentration. Whereas Harvey (1926, 1928) has recorded marked seasonal variations in the nitrate-content in the English Channel with non-detectable amounts in August, Dakin (1934) showed that at Sydney there was a much more uniform distribution throughout the year. It would appear, therefore, that marked seasonal variations in the nutrient content, with depletion of nitrate in the summer, occur mainly in inshore waters where a thermocline is set up during the summer preventing regeneration of the nutrients in the photosynthetic layer.

Marshall & Orr (1948), in carrying out experiments in Loch Craiglin on the effect of different plant nutrients on the phytoplankton, found that after adding a large excess of fertilizer a good increase in plankton was obtained, after an initial lag. At that time attached algae were abundant and these investigators believed that the needs of the algae had to be satisfied before any nutrients could be used by phytoplankton.

With marine diatoms, therefore, scientific investigations have established a direct correlation between their efflorescence and abundance, and the concentrations of nutrient salts, especially nitrates and phosphates, in sea water, and scientific literature pertaining to European, American, Antarctic and other waters furnishes numerous examples.

Parke (1948), in her studies on the British Laminariaceae, found for *Laminaria saccharina* that there were two periods of growth, a period of rapid growth between January and June/July with the most rapid growth between March and June, and a period of slow growth from July to December, but unfortunately no attempt was made to correlate this with the nutrient content of the water.

Tikhovskaya (1940) investigated the seasonal variations in the productivity and photosynthesis of *L. saccharina* in the Barents Sea. He reported maximum

growth in April with a sharp drop in June and a slight increase in October, and stated, although no figures are given, that in summer the N and P content was the smallest for the whole year.

In view of the fact that in Norway, it has been possible to correlate the presence of herring with water of a particular temperature and salinity, it was thought that, since correlation appeared to exist between the chemical composition of the algae and the physico-chemical properties of the water, it might be possible from an analysis of the water to forecast the optimum annual harvesting periods. No previous investigator, however, appears even to have attempted to correlate the seasonal changes in the chemical composition of the fixed algae with changes in the composition of the sea water.

Preparation and Analysis of Samples

Monthly samples comprising six plants of the following species of Laminariaceae were taken, two were used for dry weight determinations, and two were analysed by the methods previously employed (Black, 1948).

The inorganic nitrogen was obtained by difference, i.e. by subtracting the organic nitrogen from the total nitrogen. The total nitrogen was determined by modifying the standard Kjeldahl method using a reduction mixture containing sodium thiosulphate as a preliminary, to the ordinary digestion (Kelley, Hunter & Sterges, 1946). Any ammonium salts, however, present in the plant will be estimated as organic nitrogen (Kjeldahl).

(1) *L. saccharina* from 4 m. (low water) at Eilean Coltair, Loch Melfort.
(2) *L. saccharina* from 4 m. at Rudh-an-Aoil, Shuna Island.
(3) *L. cloustoni* from 4 m. at Cullipool, Luing Island.

The results for crude proteins, inorganic nitrogen, mannitol, laminarin, dry-weight content, fresh weight, total ash, and alginic acid are summarized in Figs. 7–14.

As the marked seasonal variations in composition occur in the fronds the results for the stipes, with the exception of the fresh weights for *L. saccharina*, have been omitted.

Discussion of Results

In comparing the variations in chemical constitution it should be borne in mind that *L. cloustoni* differs in many respects from *L. saccharina*, and it is unfortunate that the latter species was unobtainable at Cullipool.

On the west coast of Scotland, with *L. saccharina*, growth of the new frond begins much earlier than with *L. cloustoni*, and from August losses due to the shedding of the old frond of the current year may exceed the weight increment. In the autumn the rapid drop in laminarin may be due to this or it may be due to sporogenesis. On the other hand, *L. cloustoni* does not usually cast its old frond until April/May, although considerable wear has no doubt occurred

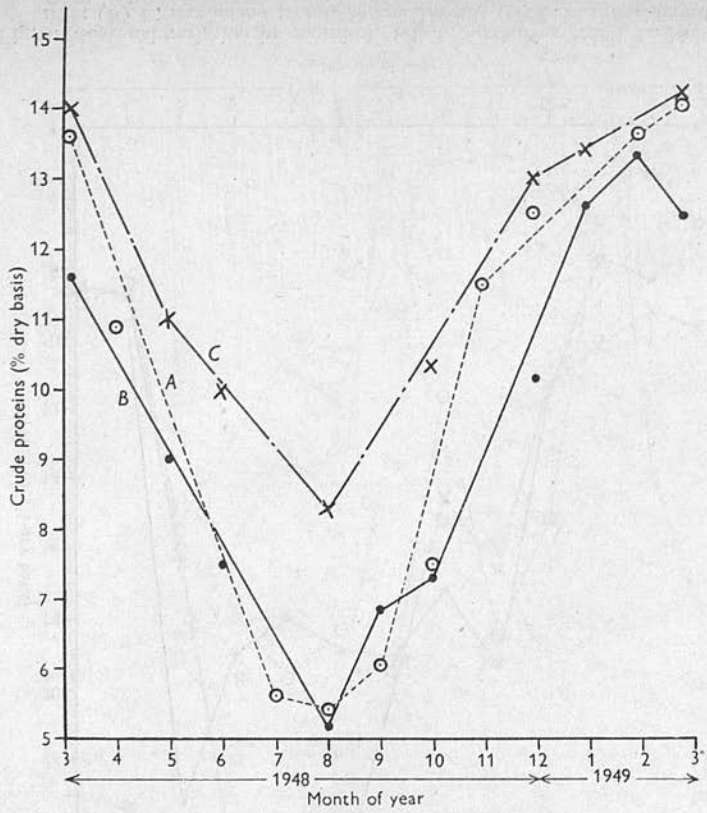

Fig. 7. Seasonal variation in crude proteins. (*A*) *L. saccharina* frond, L. Melfort; (*B*) *L. saccharina* frond, Shuna Island; (*C*) *L. cloustoni* frond, Cullipool.

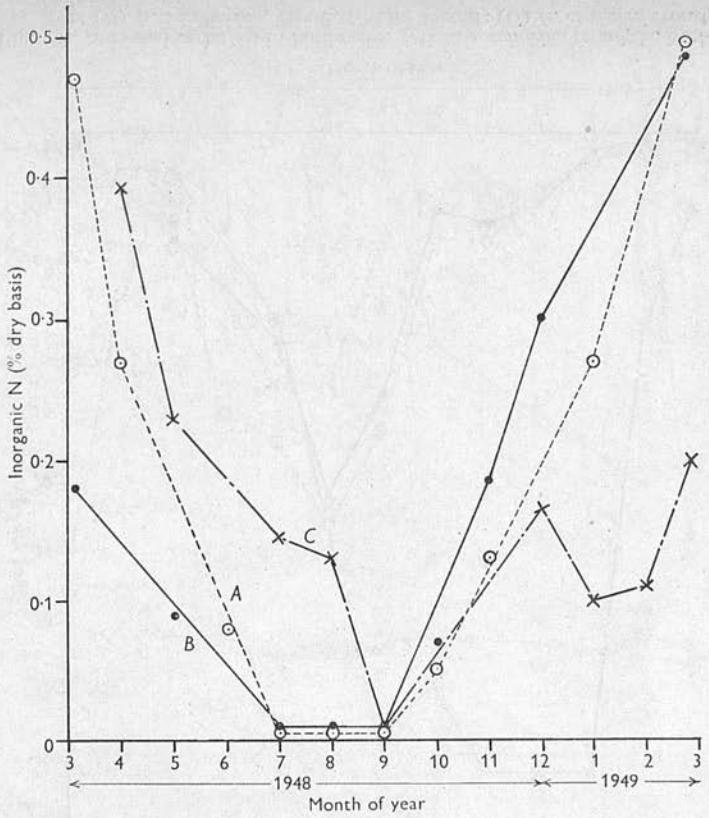

Fig. 8. Seasonal variation in inorganic nitrogen. (*A*) *L. saccharina* frond, L. Melfort; (*B*) *L. saccharina* frond, Shuna Island; (*C*) *L. cloustoni* frond, Cullipool.

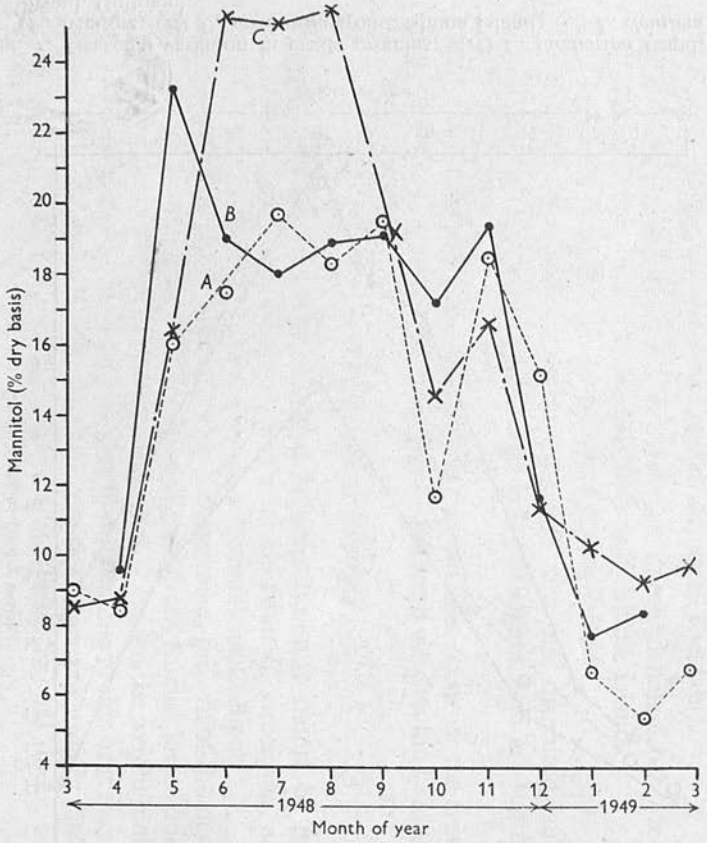

Fig. 9. Seasonal variation in mannitol. (*A*) *L. saccharina* frond, L. Melfort; (*B*) *L. saccharina* frond, Shuna Island; (*C*) *L. cloustoni* frond, Cullipool.

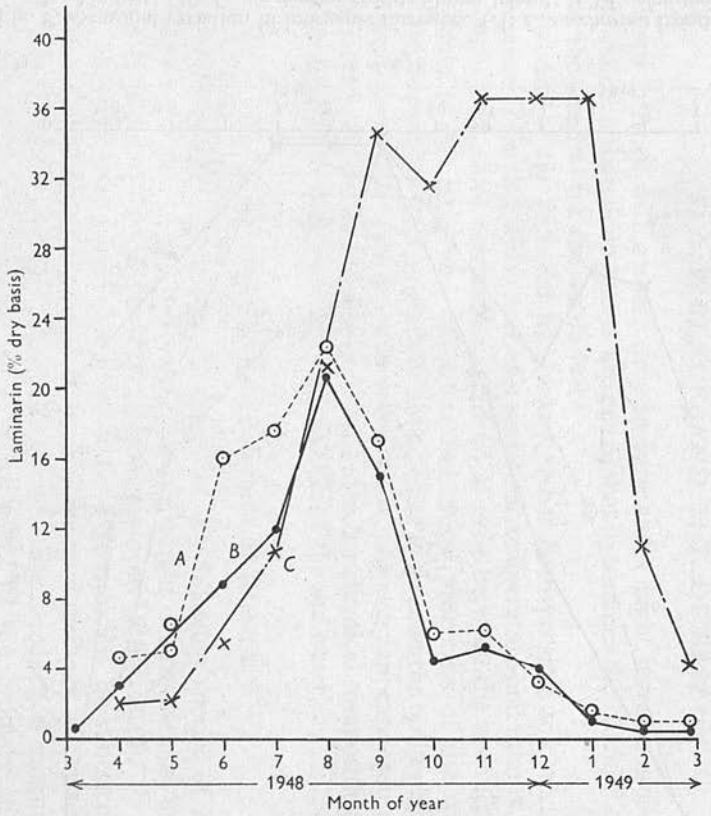

Fig. 10. Seasonal variation in laminarin. (*A*) *L. saccharina* frond, L. Melfort; (*B*) *L. saccharina*, frond, Shuna Island; (*C*) *L. cloustoni* frond, Cullipool.

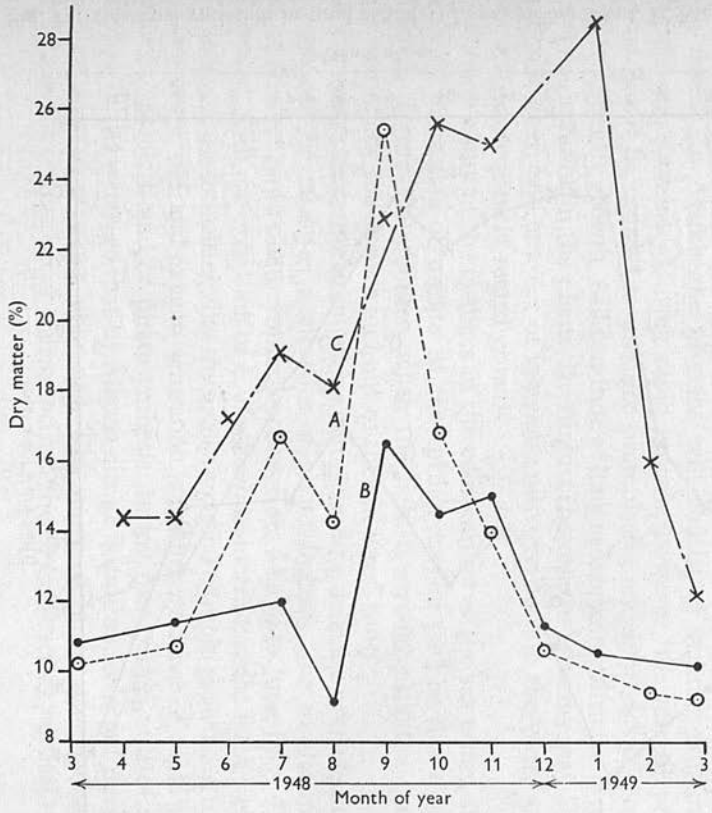

Fig. 11. Seasonal variation in dry matter. (*A*) *L. saccharina* frond, L. Melfort; (*B*) *L. saccharina* frond, Shuna Island; (*C*) *L. cloustoni* frond, Cullipool.

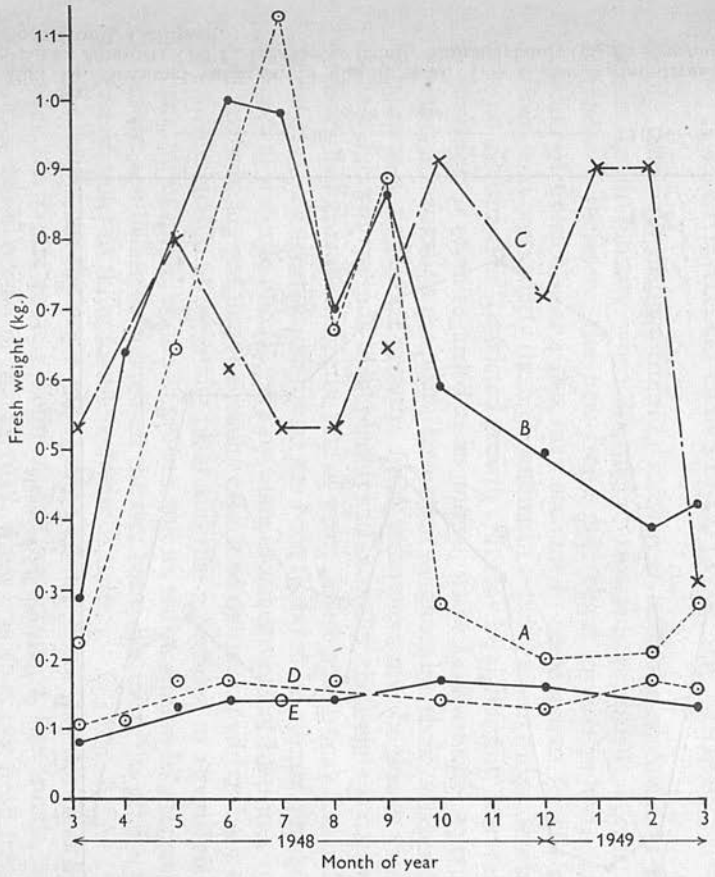

Fig. 12. Seasonal variation in fresh weight. (*A*) *L. saccharina* frond, L. Melfort; (*B*) *L. saccharina* frond, Shuna Island; (*C*) *L. cloustoni* frond, Cullipool; (*D*) *L. saccharina* stipe, L. Melfort; (*E*) *L. saccharina* stipe, Shuna Island.

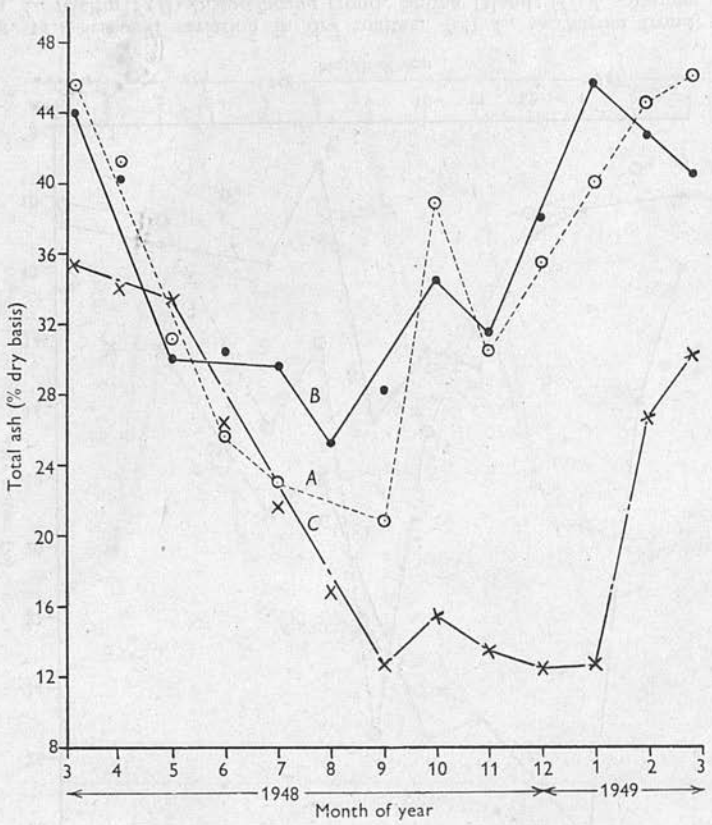

Fig. 13. Seasonal variation in total ash. (*A*) *L. saccharina* frond, L. Melfort; (*B*) *L. saccharina* frond, Shuna Island; (*C*) *L. cloustoni* frond, Cullipool.

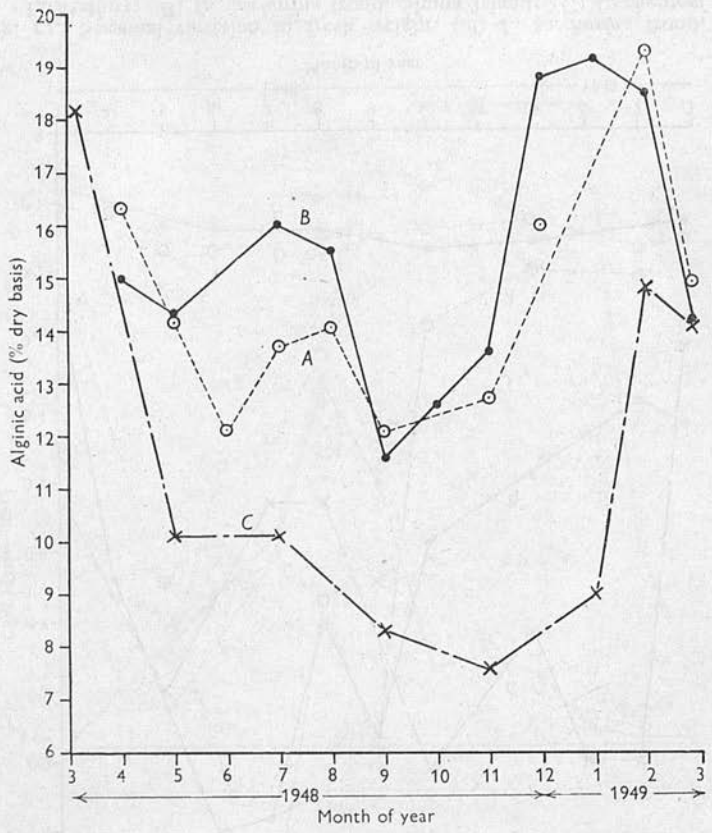

Fig. 14. Seasonal variation in alginic acid. (*A*) *L. saccharina* frond, L. Melfort; (*B*) *L. saccharina* frond, Shuna Island; (*C*) *L. cloustoni* frond, Cullipool.

before then. As distinct from *L. saccharina* laminarin is found at a high percentage in January when sporing of this species usually occurs. With *L. cloustoni*, therefore, the new frond may begin growth in water already depleted of nutrients, and this may account to some extent for the results obtained, and particularly the high laminarin content which the writers believe to be an indication of 'restricted growth'. It may be significant, however, that *L. saccharina* is often regarded as an annual, while *L. cloustoni* is a perennial.

In this investigation the plants have been weighed, but these weights cannot be taken as a measure of growth. First, differences may be due to variations in the weights of the individual plants; and secondly, the weight is determined by the rate of growth minus the rate of wear. Sporogenesis may also influence the weight, as a sporogenous frond has been found to be approximately twice as heavy as a sterile one (Report by State Oceanographical Institute, 1933).

It might be argued that the results expressed on the anhydrous basis do not give a true picture of the living plant and that a decrease in a particular constituent, for example, is due to growth and its redistribution in a larger plant. When the results are calculated on the wet basis, however, the graphs are, in general, parallel to those for the anhydrous basis.

The results, on the other hand, could be expressed per unit plant. If we consider the crude protein content, the maximum in the fronds, on the anhydrous basis, occurs in March 1948, when it is 50 g. per fresh frond. In August when it is at a minimum, on the anhydrous basis, it is 140 g. per fresh frond. In March the proteins will consist of reserve proteins and protoplasmic proteins. During rapid growth, however, the reserve proteins will no doubt be utilized to produce protoplasm; and synthesis of proteins, and consequently growth, will continue as long as inorganic nitrogen is present in the sea water or in the plant. By August the reserve proteins may all have been converted, and absence of inorganic nitrogen both in the water and the plant will consequently retard growth.

Although many of the changes in the composition of the sea water, such as the rapid utilization of nitrate in the spring, may in part be due to phytoplankton, during the summer, when the light is strong, plankton probably keep well below the surface and out of the sea-weed zone.

If carbohydrate assimilation with a corresponding decrease in crude proteins and ash can be taken as a measure of photosynthesis, the results indicate that a period of rapid photosynthesis occurs from March to June/July. During this period, with the exception of *L. cloustoni*, an increase in the fresh weight of the plants occurs indicating that this is also a period of rapid growth. A marked increase in the pH and oxygen saturation is further evidence of rapid photosynthesis. Growth and/or photosynthesis during this period utilizes all the nutrients in the water, so that by August nitrate is undetectable and phosphate is as low as 0·16–0·19 mg. atom/m.^{3}. The warming of the waters may have resulted in the setting up of a thermocline, thereby restricting vertical mixing

so that regenerated nutrients do not reach the photosynthetic layer. In September cooling of the waters results in the autumn mixing and the replenishment of the photosynthetic layer with nutrients, so that in October/November a second burst of photosynthesis but on a reduced scale occurs, resulting again in increased carbohydrate assimilation. With light, no doubt, now the limiting factor, photosynthesis slows up and the nutrients accumulate again to reach their winter maxima in January/February.

In foreign waters such as the San Juan Channel, Washington, U.S.A., where *Nereocystis luetkeana* predominates, nitrate and phosphate have been determined for a period of 5 years (Phifer & Thompson, 1937). The great velocity of the tidal currents there creates very turbulent conditions and no thermocline is set up as in our inshore waters. At all times of the year the phosphate concentration was always sufficient for plant growth and could never be considered as a limiting factor. From a maximum of about 3 mg. atoms/m.^{3} in December it decreased to 0·8 mg. atom/m.^{3} in August, and this minimum value is considerably greater than our winter maximum. Nitrate, at a maximum of about 35 mg. atoms/m.^{3} in December/January, dropped to 0·5 mg. atom/m.^{3} in August, a minimum value of the order of magnitude of our winter maximum.

Although it appears that some correlation exists between the seasonal variation in the composition of the British Laminariaceae and variations in the sea water, it does not appear evident to the authors how, by taking and analysing a sample of sea water, it is possible to predict the appropriate time of harvesting, when a desired constituent will be at a maximum. A 4-year investigation (Black, 1948, and in the press) of the seasonal variation of some of the common British Laminariaceae has provided that information. To establish the correlations between the physico-chemical properties of the water and the fixed algae would require quite extensive work over a period of years, and once established it would still be necessary to keep sampling the sea water at intervals to ensure that the prognostication was being followed as expected.

In March the fronds of the Laminaria are low in mannitol and laminarin (Figs. 9 and 10) and high in proteins and inorganic nitrogen (Figs. 7 and 8), after the carbohydrates have been used up during the winter in respiration and probably in the synthesis of proteins, which are believed to be the product of a 'dark' synthesis. In March also, the alginic acid content is at a maximum (Fig. 14), but the frond is exceedingly small (Fig. 12) and high in water content (Fig. 11), while the cell sap contains much mineral matter (Fig. 13). During the winter months the rate of photosynthesis has been very low, if any photosynthesis occurs at all, and consequently the surrounding sea water is high in nitrates and phosphates.

In March/April a marked increase in photosynthesis occurs, accompanied by an increase in the fresh weight of the plant and an increase in the mannitol, while a decrease occurs in the crude proteins and inorganic nitrogen, all

indicating that vigorous growth has commenced. Simultaneously, in the surrounding sea water a drop occurs in the nitrate and phosphate content as a result of their utilization by algae and other forms of marine life. At the same time an increase in the pH and oxygen saturation content occurs, which is further evidence of a marked increase in photosynthesis.

While a drop of only 0·03 mg. atom/m.^{3} occurs in the phosphate content of the water at Shuna Island, the phosphate content at Loch Melfort falls 0·20 mg. atom/m.^{3} during March/April. The change in the nitrate content is more marked during this period; at Shuna Island it falls from 6·1 to 3·1 mg. atoms/m.^{3}, while at Loch Melfort it decreases from 6·4 to 0·7 mg. atom/m.^{3}.

During May photosynthesis continues. *Laminaria saccharina* increases in weight, but a decrease in the weight of *L. cloustoni* occurs, no doubt due to the shedding of the old frond at this time. Mannitol shows an increase, and a decrease occurs in the crude proteins, inorganic nitrogen, alginic acid and ash. In the sea, the temperature of which is gradually increasing, the pH and oxygen saturation content show an increase, and a marked drop in the nitrate content at Cullipool and Shuna Island occurs, while phosphate decreases to 0·33, 0·26 and 0·19 mg. atom/m.^{3} at Cullipool, Shuna Island and Loch Melfort respectively.

In June there is a suggestion of a 'falling off' in the rate of photosynthesis. The fronds of *L. saccharina* from Shuna Island show a decrease in mannitol, but an increase in fresh weight, however, occurs in both samples of *L. saccharina*, while a decrease in the weight of *L. cloustoni* is recorded. The crude proteins, inorganic nitrogen, ash and alginic acid contents show a further decrease with an appreciably higher protein figure for *L. cloustoni* at Cullipool. While the pH and oxygen saturation of the water remain constant, a further drop in the nitrate content occurs, while phosphate is between 0·16 and 0·20 mg. atom/m.^{3}.

In July/August there is evidence of a marked change in the rate of growth and/or the rate of photosynthesis. Laminarin shows an increase but this is characteristic of 'restricted' growth, mannitol remains relatively constant, but a drop occurs in the dry matter and fresh weight of *L. saccharina*. The water analysis shows the temperature rising to a maximum of 13°C. at the three localities. In July, nitrate and phosphate are at a minimum, the nitrate being 0·9 mg. atom/m.^{3} at Cullipool, while at Shuna Island and Loch Melfort it is undetectable in the water, and phosphate is between 0·16 and 0·20 mg. atom/m.^{3}.

The pH and dissolved oxygen saturation, which reach maxima in July, fall rapidly in August, indicating reduced photosynthesis in the water. The ash content shows a slight decrease, but a marked increase occurs in the alginic acid content.

The months of July and August appear to be critical ones, and in all our work, and in the work of other investigators, comment has been passed on the change

in composition and rate of growth which occurs at this time. There may be other influencing factors, but the water analysis shows that lack of nutrients must be a limiting factor. Although low concentrations of nitrate, for example, exist in the water at Loch Melfort in April the weed still contains a reserve of inorganic nitrogen, so that it is not until July, with the exception of *L. cloustoni* which always exhibits a lag, that nitrate is undetectable in the plant and the water.

Temperature may also be an influencing factor, as it reaches a maximum of 13°C. at this time of the year. The intensity of respiration and assimilation increases with temperature, but the effect on respiration is considerably greater than on assimilation. Consequently with increasing temperature, the surplus will eventually disappear and growth will cease.

In September/October a rise occurs in the protein content of all the plants, coinciding with an increase in the nitrate and phosphate contents of the water. This is the result of the autumn cooling of the water bringing about the breakdown of the thermocline, giving vertical mixing and regeneration of nutrients in the photosynthetic zone. A marked increase in the pH of the water signifies a second 'burst' of photosynthesis, less intense however, than the spring outburst. An increase in the fresh weight and dry-matter content occurs, but a considerable drop in the laminarin occurs which might be the result of further growth.

In November/December, although a drop in the dry matter and fresh weight of the plants occurs, there is a rise in the mannitol, laminarin and crude protein contents and a decrease in the ash, indicating that the rate of assimilation still exceeds the rate of respiration. The rapid increase since August in the nitrate concentration is checked, indicating its utilization.

From December to March photosynthesis proceeds at a low rate as light may now be the limiting factor. Little change occurs in the pH, while the carbohydrates are now utilized in growth and respiration at a greater rate than they are assimilated.

Nitrate and phosphate increase to a maximum in January/February and then begin to decrease again, the exception being the high phosphate figures for Shuna Island and Loch Melfort in December.

The large store of inorganic nitrogen present in the fronds during the winter and early spring is rather surprising and is worthy of further investigation.

These seasonal variations in composition appear to be characteristic of most forms of marine life. For example, Daniel (1922) investigated the seasonal variation in chemical composition of the mussel and found from May onwards a decrease in the proteins, on the dry ash free basis, reaching a minimum in September/October and then rising to a maximum the following March. The carbohydrates showed a steady increase from March, reaching a maximum in September/October, with a tendency to form a second maximum in December, and then a rapid decrease until March.

Acknowledgements

The work described in this report forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association, to whom the authors are indebted for permission to publish. They also wish to thank the staffs at the Marine Stations at Aberdeen, Millport and Plymouth for their very valuable help, and for the loan of essential apparatus.

The assistance of Miss B. Graham and Mr W. Cornhill in the analytical work is also acknowledged.

Summary

Monthly samples of the Laminariaceae, *L. saccharina* and *L. cloustoni*, from three localities on the Argyllshire coast have been taken from March 1948 to March 1949, and analysed for dry matter, ash, mannitol, laminarin, crude proteins, inorganic nitrogen and alginic acid, and the seasonal variation in these constituents correlated with the changes in composition of the sea water.

The results show that a correlation does exist and that a period of rapid photosynthesis occurs from March to June/July, but is restricted in July/August when nitrate is undetectable in the water and phosphate is as low as 0·16–0·20 mg. atom/m.^{3}. The replenishment of the photosynthetic layer with nutrients is retarded in July/August, apparently due to the warming of the inshore waters, which may set up a thermocline restricting vertical mixing.

The autumn cooling of the uppermost waters facilitates vertical mixing, regenerating the nutrients in the photosynthetic region, and a second burst of photosynthesis at a reduced rate from the spring 'outburst' occurs in October/November.

References

Atkins, W. R. G., 1923. The phosphate content of fresh and salt waters in its relationship to the growth of the algal plankton. *Journ. Mar. Biol. Assoc.*, Vol. xiii, pp. 119–50.

—— 1926. The phosphate content of sea water in relation to the growth of the algal plankton. Part III. *Journ. Mar. Biol. Assoc.*, Vol. xiv, pp. 447–67.

—— 1928. Seasonal variations in the phosphate and silicate content of sea water during 1926 and 1927 in relation to the phytoplankton crop. *Journ. Mar. Biol. Assoc.*, Vol. xv, pp. 191–205.

—— 1930. Seasonal variations in the phosphate and silicate content of sea water in relation to the phytoplankton crop. Part V. November 1927 to April 1929, compared with earlier years from 1923. *Journ. Mar. Biol. Assoc.*, Vol. xvi, pp. 821–52.

—— 1932. Nitrate in sea water and its estimation by means of diphenylbenzidine. *Journ. Mar. Biol. Assoc.*, Vol. xviii, pp. 167–92.

PHIFER, LYMAN D. & THOMPSON, THOMAS G., 1937. Seasonal variations in the surface waters of San Juan Channel during the five year period, January 1931 to December 30, 1935. *Journ. Mar. Research*, Vol. I, pp. 34–59.

REPORT BY STATE OCEANOGRAPHICAL INSTITUTE OF MOSCOW, 1933. Expedition for the exploration of the iodine-bearing algae along the White Sea and Murmansk Coast. *Trans. Oceanographical Institute*, Vol. 3, No. 3, pp. 5–124.

RUSSELL, F. S., 1935. The seasonal abundance and distribution of the pelagic young of teleostean fishes caught in the ring trawl in offshore waters in the Plymouth area. Part II. *Journ. Mar. Biol. Assoc.*, Vol. XX, pp. 147–79.

—— 1936*a*. The seasonal abundance of the pelagic young of teleostean fishes in the Plymouth area. Part III. The Year 1935, with a note on the conditions as shown by the occurrence of plankton indicators. *Journ. Mar. Biol. Assoc.*, Vol. XX, pp. 594–604.

—— 1936*b*. The importance of certain plankton animals as indicators of water movement in the western end of the English Channel. *Cons. Int. Explor. Mer, Rapp. Proc. Verb.*, Vol. C, Part 3, pp. 7–10.

THOMPSON, THOMAS G. & ROBINSON, REX J., 1933. The sea water of the Puget Sound region. *Proc. 5th Pac. Sci. Congress, Canada*, Vol. III, pp. 2101–2107.

TIKHOVSKAYA, Z. P., 1940. Seasonal variations in the productivity and photosynthesis of *Laminaria saccharina* in the Dalne-Zelenetz Bay of the Barents Sea. *C.R. Acad. Sci., U.R.S.S.*, Vol. XXIX, pp. 120–4.

WATTENBERG, H., 1937. Critical review of the methods used for determining nutrient salts and related constituents in salt water. *Cons. Int. Explor. Mer, Rapp. Proc. Verb.*, Vol. CIII, pp. 1–25.

Reprinted from the Journal of the Society of Chemical Industry, 1950, 69, pp. 161–165

THE EFFECT OF THE DEPTH OF IMMERSION ON THE CHEMICAL CONSTITUTION OF SOME OF THE SUB-LITTORAL SEAWEEDS COMMON TO SCOTLAND

By W. A. P. BLACK

An investigation into the variations in chemical constitution of the common British Laminariaceae, *L. cloustoni*, *L. digitata* and *L. saccharina*, collected at depths of 1–24 m., has been carried out in Orkney. At certain times of the year variations in chemical composition, in addition to those due to the recognized seasonal variations, have been found to occur in the fronds with very little variation in the stipes. In general, in the fronds an increase in mannitol occurs with depth, reaching a maximum at 6–10 m., indicating that maximum photosynthesis probably occurs at that depth, in agreement with the observations of previous workers. Below 10 m., the mannitol decreases again. Laminarin decreases progressively with depth, being at a maximum at 3–6 m., and is absent in samples from a depth below 16 m.

Introduction

In a previous communication[1] the variation in chemical composition of *Laminaria cloustoni* taken at depths of 1–13 m. has been reported. The present paper summarizes the results obtained for the three sub-littoral seaweeds, *L. cloustoni*, *L. digitata* and *L. saccharina*, samples of which were taken in Orkney at depths of from 1–24 m. (low-water ordinary spring tides). The results show that at certain times of the year variations in chemical composition, in addition to variations due to the recognized seasonal change, occur with the depth of immersion of the plant. The most marked variations occur in the summer when the energy of light is at a maximum and therefore it would appear that light is the chief limiting or controlling factor.

In a recent publication[2] it has been shown that correlation exists between the chemical composition of the littoral weeds and the depth at which they grow. Haas and Hill[3] and Russel-Wells[4] included *L. digitata* in their investigations, which were concerned chiefly with the variation in the fat content. The author is not aware of work of any kind which has been carried out on the variation in the chemical composition of the sub-littoral weeds with depth of immersion nor are there any references even to the possibility of such a variation existing.

In the littoral zone Gail[5] has shown that light is a determining factor in fixing the lower limit at which the Fucaceae can grow. Moore[6] has stated that the rate of photosynthesis decreases in July when the light is too strong, while other workers[7] investigating the photosynthesis of algae at different depths in Trout Lake, Wisconsin, found a lower rate at the surface due to excessive light. Similar work has also been carried out by Schomer and Juday[8]; they measured the oxygen production, in three-hour periods, by two species of algae growing in nutrient solutions contained in bottles which were immersed in four different lakes representing types lying between those possessing clear transparent water to those of deep brown colour. The maximum oxygen production was found at the surface on dark cloudy days, and at a certain distance below the surface on bright clear days. Moore, Whitley and Webster[9] also found that various brown algae carry on photosynthesis most actively not at the surface, but at a reduced illumination.

Tikhovskaya[10] investigated the seasonal variations in the productivity and photosynthesis of *L. saccharina* at depths of 0, 4 and 10 m. in the Dalne–Zelenetz Bay of the Barents Sea and found a decrease in photosynthesis under conditions of high temperature and exposure, the results indicating that both temperature and light optimum of this plant are low. Parke,[11] in her observations on the growth of the Laminariaceae, has found in *L. saccharina* a variation in frond development with depth of submergence. Stipe and holdfast development during the third period of rapid growth also showed a variation with depth of submergence of the plants.

A resumé with extensive bibliography on the relations between photosynthesis, submarine daylight, and the vertical distribution of some of the marine algae, has been given by Levring.[12] Besides reviewing the work of other investigators in this field, he gives the results of his own investigations into the photosynthetical effect of different spectral ranges, and the effect of radiant energy on photosynthesis at different depths.

Experimental

Preparation of samples.—The samples were all taken by means of a spring grab, operated from a boat in an area between Strombery Point and Elwick in Orkney, the first set of samples on August 13, 1946, the second set on June 5, 1947, and the third set on July 30, 1947, while the regular monthly samples were taken on the first or second day of each month. Samples of *L. cloustoni* were also taken at Sanday Sound on July 6, 1947. The plants were draped over ropes in a shed heated by two electric fires and dried at a temperature of approximately 20° C. for 72 hr. The dried plants were then despatched to Edinburgh, where, after further drying at 25–30° C. for 24 hr. they were ground in a Christy and Norris No. 8 Laboratory Mill fitted with a $\frac{1}{64}$-in. perforated screen giving a product which practically all passed 90 mesh.

The analytical methods used were those previously employed by the writer.[1] The results calculated on the anhydrous basis are given in Figs. 1–8. The high mannitol content (36·7%) obtained by the standard titration method was checked by extracting the mannitol with butanol. 35·8% was extracted in this way, while the weed residue contained 1·6% (by titration). In carrying out the alginic acid estimations it was observed that there was a progressive increase in the depth of colour of the sodium carbonate extracts from a pale yellow solution at 2 m. to a black solution at 14 m. There also appeared to be a variation in the viscosity of the alginic acid with depth, the sodium carbonate extract at 2 m. setting to a gel, whereas at 12 and 14 m. it was quite fluid.

Discussion of results

General.—In general, at certain times of the year a variation in the chemical constitution of the frond of the three *Laminariae*, *L. cloustoni*, *L. digitata* and *L. saccharina*, occurs with depth of immersion, whereas the composition of the stipes remains relatively constant.

In the three *Laminariae*, mannitol increases with depth of immersion to a maximum at 6–10 m. and then decreases. If mannitol is the primary product of photosynthesis, or even if it can be taken as an indication of the amount of photosynthesis, it follows, in agreement with the results of previous investigators, that the maximum photosynthesis occurs at a depth of 6–10 m. and not at the surface. This is also in agreement with the theory of complementary chromatic adaptation, brown algae being better adapted for depths of 5–15 m. where the red light is absent, Levring (*loc. cit.*) having shown that the spectral distribution of light energy in different layers of water is of great importance to the problem of carbohydrate assimilation. Brown algae such as *L. digitata* showed a strong assimilation in green light.

The results for the monthly samples of *L. cloustoni*, taken at depths of 4, 8 and 12 m. during 1948 show very little variation until August 1. It may be significant that in 1948 Orkney had a very poor summer with practically no sunshine in marked contrast to the good summers of 1947 and 1946. The absence of sunshine, therefore, in 1948 would not be so conducive to variations in the rate of photosynthesis with depth. Atkins[13] has shown, for example, that the illumination to which

algae are subjected varies with the season of the year and with the clearness of the water, and is generally at a maximum from May to July. Colin and Ricard[14] and Lunde[15] attempted to correlate the composition of the *Laminariae* with hours of sunshine, and concluded that the amount of sunshine was at least an influencing factor in the synthesis of laminarin. This is in agreement with the results of this investigation, also, the percentage of laminarin has been found to decrease with depth of immersion, and has not been found in plants growing at depths from 20–24 m. The possibility, however, of laminarin occurring later in the year cannot be excluded.

It is significant from the results of this investigation that when variations in composition occur with depth, a correlation exists between the mannitol and the ash, or the mannitol and the laminarin. Thus when the laminarin content is low, as it was in *L. digitata*, *L. saccharina* and *L. cloustoni* in June, 1947, an increase in mannitol with depth is accompanied by a decrease in ash. If the conditions are favourable for the synthesis of laminarin, however, the ash remains constant with depth and correlation exists between the mannitol and the laminarin down to a certain depth.

Slight variations in composition may be due to differences in the individual plants, but previous work has shown that these differences are very small, and this is supported by the results of this investigation (Fig. 7), where for the greater part of the year the fronds from two plants of *L. cloustoni* from depths of 4, 8 and 12 m. show very slight differences in ash, mannitol and alginic acid. Differences in composition, however, could occur as the result of a diurnal lag with depth; that is, if samples at 4 m. had been taken at 10 hr., and samples at 6 m. at 12 hr. different results might have been obtained. In this investigation the samples from the various depths were taken within one hour so that any diurnal variation is almost eliminated.

In an investigation of this problem, however, it is essential to correlate the effect of depth of immersion on chemical constitution with (i) any variations in the chemical and physical properties of the water, (ii) variations in the rate of growth and possibly the times of reproduction, (iii) measurements of the energy and quality of light at various depths, and (iv) observations on the factors affecting the penetration of light.

Growth depends on the ratio of assimilation to respiration, and any difference in the rate of growth with depth will be due to variations in the assimilatory surplus. The intensity of respiration and assimilation increases with temperature, but the influence on respiration is considerably greater than on assimilation, and with increasing temperature the surplus will eventually disappear and growth will cease. In the summer months the temperature of our waters decreases with depth so that temperature could also account for a difference in the assimilatory surplus and thereby influence the rate of growth and the chemical composition.

The compensation point, i.e. the depth at which plants lose more by respiration than they gain by photosynthesis, depends on the intensity of illumination, and factors affecting the transparency of the water. This point will undergo both diurnal and seasonal variations, which will affect growth, and consequently the chemical composition of the algae.

Laminaria cloustoni

The results for *L. cloustoni*, taken in August 1946,[1] show that marked variations in the chemical composition of the fronds occur with depth, with only slight variations in the stipes. In the fronds, for example, mannitol 19·4% at 1 m. increases to 36·7% at 8 m. and then decreases. Samples of *L. cloustoni* fronds, taken in June 1947, when the laminarin content is low, show a maximum mannitol content of 32% at 6 m. decreasing progressively to 19·5% at 15 m. (Fig. 1). In the stipes the

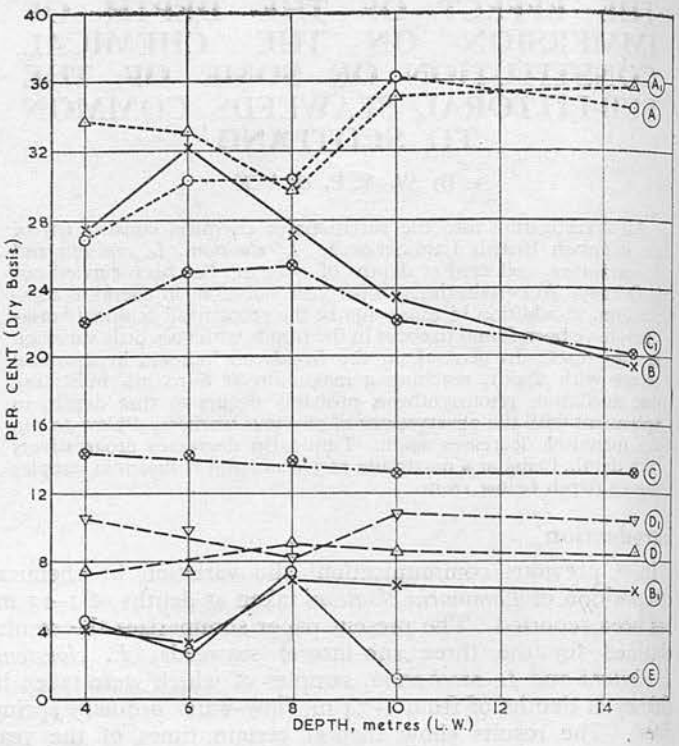

FIG. 1. *Variation in composition in* L. cloustoni, *Orkney, June,* 1947

A Ash in the fronds
B Mannitol in the fronds
C Alginic acid in the fronds
D Proteins in the fronds
E Laminarin in the fronds
A1 Ash in the stipes
B1 Mannitol in the stipes
C1 Alginic acid in the stipes
D1 Proteins in the stipes

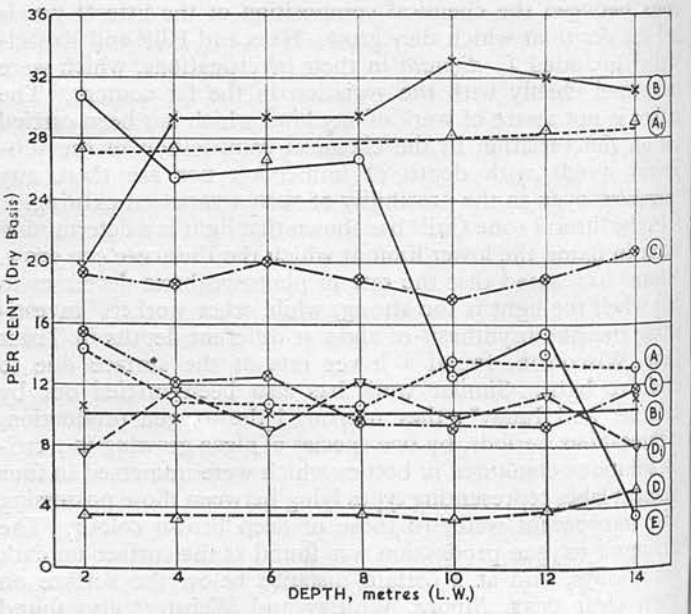

FIG. 2. *Variation in composition in* L. cloustoni, *Orkney, July,* 1947

A Ash in the fronds
B Mannitol in the fronds
C Alginic acid in the fronds
D Proteins in the fronds
E Laminarin in the fronds
A1 Ash in the stipes
B1 Mannitol in the stipes
C1 Alginic acid in the stipes
D1 Proteins in the stipes

mannitol graph is approximately the inverse of the protein graph, and the ash graph the inverse of the alginic acid graph. Samples taken in late July, when the laminarin is high (Fig. 2), indicate again an increase in mannitol, in the frond, with depth

Table I

Variations in the chemical composition of L. cloustoni, *Sanday Sound, Orkney, July* 1947 (*dry basis*)

Sample	Depth (L.W.), m.	Total ash %	Iodine %	Crude proteins, %	Mannitol %	Laminarin %	Alginic acid %
Frond	18	25·6	0·91	5·6	26·8	1·1	12·4
Stipe		25·7	1·00	7·9	8·4	< 1·0	18·2
Frond	20	24·9	1·07	7·4	21·5	0·6	12·2
Stipe		23·0	1·01	7·8	10·7	< 1·0	19·6
Frond	24	26·4	0·80	7·9	24·5	1·1	12·4

the maximum occurring at 10 m. (33%). A decrease in laminarin occurs from 30·8% at 2 m. to 3·5% at 14 m., while the composition of the stipes remains relatively constant.

In Sanday Sound where the environment, a sandy substratum, is different from that off Strombery Point, where we have a rocky substratum, the mannitol in the fronds at 18, 20 and 24 m. is lower than in the sample from 14 m. at the latter habitat, and laminarin is present to the extent of only 1% or less. (Table I.)

Regular monthly samples of *L. cloustoni*, taken at depths of 4, 8 and 12 m. (Figs. 7 and 8), show that in 1948 up to July very little variation occurs in the mannitol, total ash, and alginic acid, while there is a progressive decrease in the laminarin content with depth.

At the beginning of August variations in the mannitol content with depth first became evident, and it is unfortunate that the sampling was discontinued as further samples might have shown greater variations, which had been delayed owing to lack of sunshine.

In June 1947, the iodine content shows very little variation with depth (0·63–0·77%), but in July, marked variations again occur with a progressive increase in the frond from 0·31% at 2 m. to 1·46% at 14 m. At present there is no explanation as to why the iodine content should increase with depth. In the littoral zone iodine has also been found to increase with depth of immersion.[2]

Laminaria digitata

In the fronds in June (Fig. 3), the mannitol increases from 23% at 2 m. to 26% at 8 m., and then decreases to 13% at 14 m., while the laminarin remains low. If we compare the figures at 2 and 12 m., a considerable drop in the mannitol occurs, 23·2% to 15·5%, while the ash shows only a slight increase, 28·2% to 31·3%.

In the fronds in July (Fig. 4), mannitol increases from 19·7% at 4 m. to 37·1% at 10 m., and then decreases to 31% at 16 m., while the laminarin again remains relatively constant (1–6%). It is significant from these results that, in *L. digitata* in June, the ash and mannitol increase together to a depth of 8 m. and then the ash increases with depth as the mannitol decreases. In July the mannitol increases from 19·7% at 4 m. to 37·1% at 8 m., while the ash decreases from 34·9% to 20·3%. This follows the seasonal variation in the *Laminariae* where an increase in mannitol is accompanied by a decrease in ash, the 'mechanism' resembling one of isotonic substitution.

In June in *L. cloustoni* when the percentage of laminarin is low (Fig. 1) the same correlation exists between the mannitol and the ash. In July and August, however, when conditions, particularly in *L. cloustoni*, appear to favour the synthesis of laminarin, equilibrium exists between the mannitol and the laminarin and the ash remains constant with depth.

There is no satisfactory explanation, at present, why laminarin should accumulate to such an extent in *L. cloustoni* and

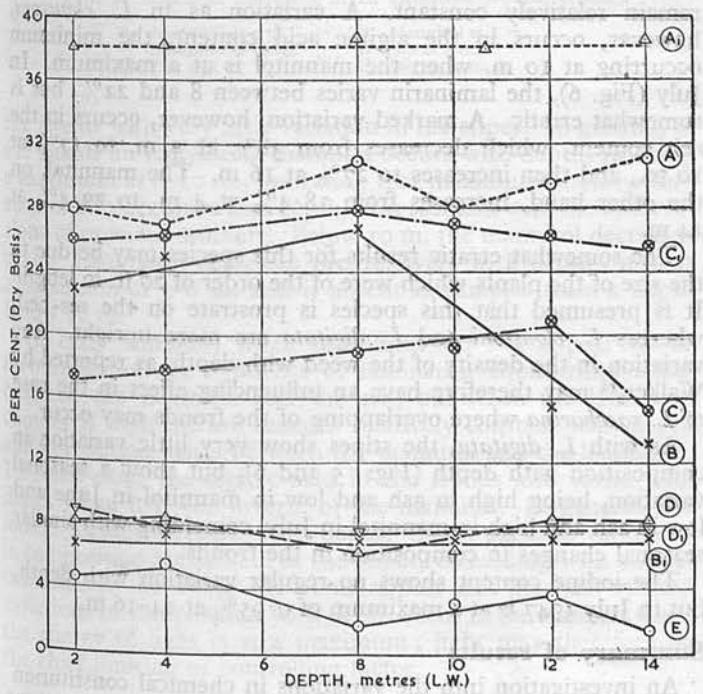

FIG. 3. *Variation in composition of* L. digitata, *Orkney, June,* 1947

A Ash in the fronds
B Mannitol in the fronds
C Alginic acid in the fronds
D Proteins in the fronds
E Laminarin in the fronds
A_1 Ash in the stipes
B_1 Mannitol in the stipes
C_1 Alginic acid in the stipes
D_1 Proteins in the stipes

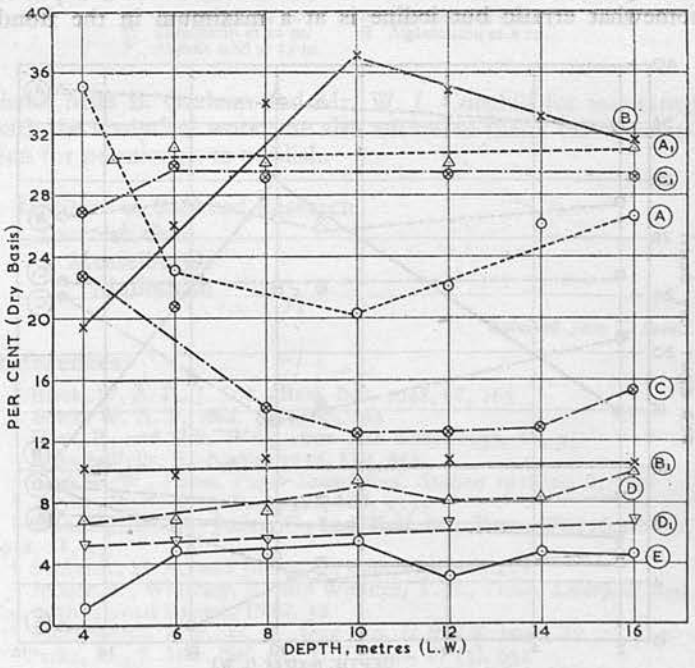

FIG. 4. *Variation in composition of* L. Digitata, *Orkney, July,* 1947

A Ash in the fronds
B Mannitol in the fronds
C Alginic acid in the fronds
D Proteins in the fronds
E Laminarin in the fronds
A_1 Ash in the stipes
B_1 Mannitol in the stipes
C_1 Alginic acid in the stipes
D_1 Proteins in the stipes

not in *L. digitata*. Work in progress on the seasonal variation in the *Laminariae* off the west coast of Scotland is also in agreement; e.g. laminarin is absent for the greater part of the year from *L. digitata* at Atlantic Bridge, and is present to the extent of 36% in *L. cloustoni* fronds at Cullipool. In this case environment appears to play a big part, rapid growth at Atlantic Bridge giving giant *L. digitata* plants. Where we have vigorous growth as at Atlantic Bridge, or in the case of the vigorous growing annual *Saccorhiza bulbosa*, laminarin is absent or at a low percentage. In Loch Melfort, consequently, we find small plants of *L. digitata*, high in laminarin. It would be interesting to transplant specimens of *L. cloustoni* from Cullipool or *L. digitata* from Loch Melfort to Atlantic Bridge and watch the effect of change of environment on growth and chemical constitution.

In Orkney, correlation will also exist between the rate of growth and the laminarin content, and it may not be that in early summer physical or chemical conditions are unfavourable for the synthesis of laminarin, but simply that it is used during this period of rapid growth. Parke (*loc. cit.*) has found a slowing down in the rate of growth in July/August, but if growth in *L. digitata* and *L. saccharina* still continues throughout the summer at a greater rate than in *L. cloustoni*, and this appears to be the case from observations in taking the samples, then this would explain why *L. cloustoni* is higher in this carbohydrate. Reproduction, however, may also have some influence on the laminarin content.

Until more is known about the metabolism of the *Laminariae* and the part played by laminarin, no satisfactory explanation can be given.

At any particular time of the year (Figs. 3 and 4), very small differences occur in the composition of *L. digitata* stipes with depth. These graphs, however, show clearly that some seasonal variation occurs, the ash which is 38% in June falling to approximately 30% in July, and the mannitol increasing from 7% in June to 10% in July coinciding with a rise in mannitol and a fall in ash in the fronds.

In June the variation in the iodine content with depth is somewhat erratic but iodine is at a maximum in the frond

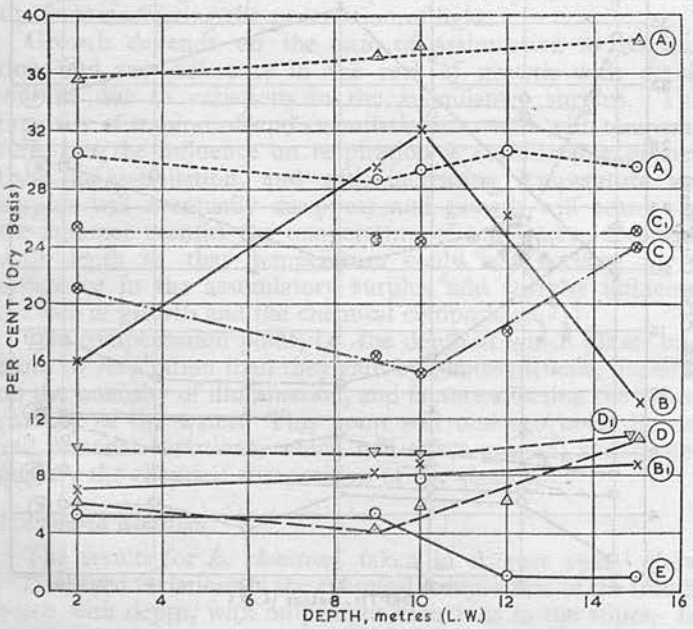

FIG. 5. *Variation in composition in* L saccharina, *Orkney, June*, 1947

A Ash in the fronds
B Mannitol in the fronds
C Alginic acid in the fronds
D Proteins in the fronds
E Laminarin in the fronds
A_1 Ash in the stipes
B_1 Mannitol in the stipes
C_1 Alginic acid in the stipes
D_1 Proteins in the stipes

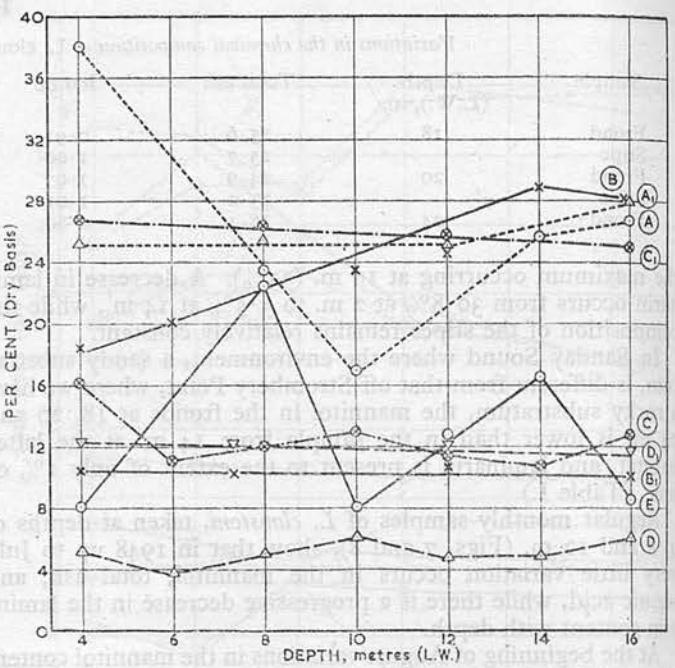

FIG. 6. *Variation in composition in* L. saccharina, *Orkney, July*, 1947

A Ash in the fronds
B Mannitol in the fronds
C Alginic acid in the fronds
D Proteins in the fronds
E Laminarin in the fronds
A_1 Ash in the stipes
B_1 Mannitol in the stipes
C_1 Alginic acid in the stipes
D_1 Proteins in the stipes

(1·31%), and the stipe (0·90%), at 14 m. In July the maximum iodine content (1·33%) in the fronds occurs again at 14 m.

Laminaria saccharina

In the fronds in June (Fig. 5), the mannitol increases from 16% at 2 m. to 32% at 10 m., while the ash and laminarin remain relatively constant. A variation as in *L. cloustoni*, however, occurs in the alginic acid content, the minimum occurring at 10 m. when the mannitol is at a maximum. In July (Fig. 6), the laminarin varies between 8 and 22%, but is somewhat erratic. A marked variation, however, occurs in the ash content, which decreases from 38% at 4 m. to 17% at 10 m., and then increases to 27% at 16 m. The mannitol, on the other hand, increases from 18·4% at 4 m. to 29·4% at 14 m.

The somewhat erratic results for this species may be due to the size of the plants which were of the order of 20 ft. in length. It is presumed that this species is prostrate on the sea-bed, whereas *L. cloustoni* and *L. digitata* are more upright. Any variation in the density of the weed with depth, as reported by Walker,[14] may therefore have an influencing effect in the case of *L. saccharina* where overlapping of the fronds may occur.

As with *L. digitata*, the stipes show very little variation in composition with depth (Figs. 5 and 6), but show a seasonal variation, being high in ash and low in mannitol in June and low in ash and high in mannitol in July, coinciding with similar seasonal changes in composition in the fronds.

The iodine content shows no regular variation with depth, but in July 1947 is at a maximum of 0·95% at 14–16 m.

Summary of results

An investigation into the variations in chemical constitution of the common British Laminariaceae, *L. cloustoni*, *L. digitata* and *L. saccharina*, collected at depths of 1 to 24 m., has been carried out in Orkney. At certain times of the year variations in chemical composition, in addition to those due to the recognized seasonal variations, have been found to occur in

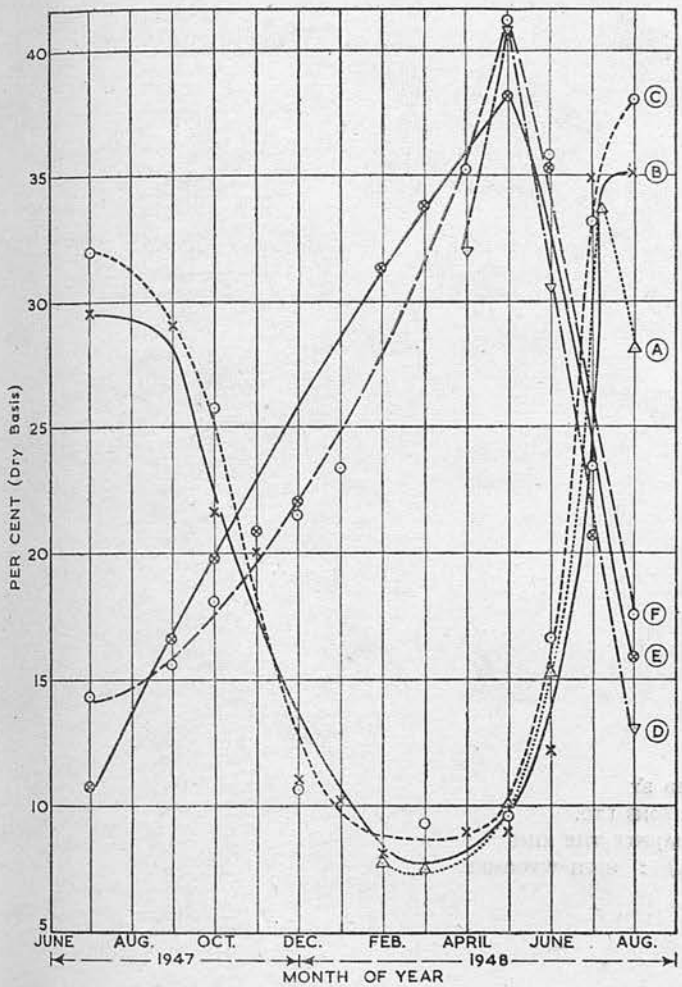

FIG. 7. *Seasonal variation in* L. cloustoni *fronds, Orkney*

A Mannitol at 4 m. B Mannitol at 8 m.
C Mannitol at 12 m. D Ash at 4 m.
E Ash at 8 m. F Ash at 12 m.

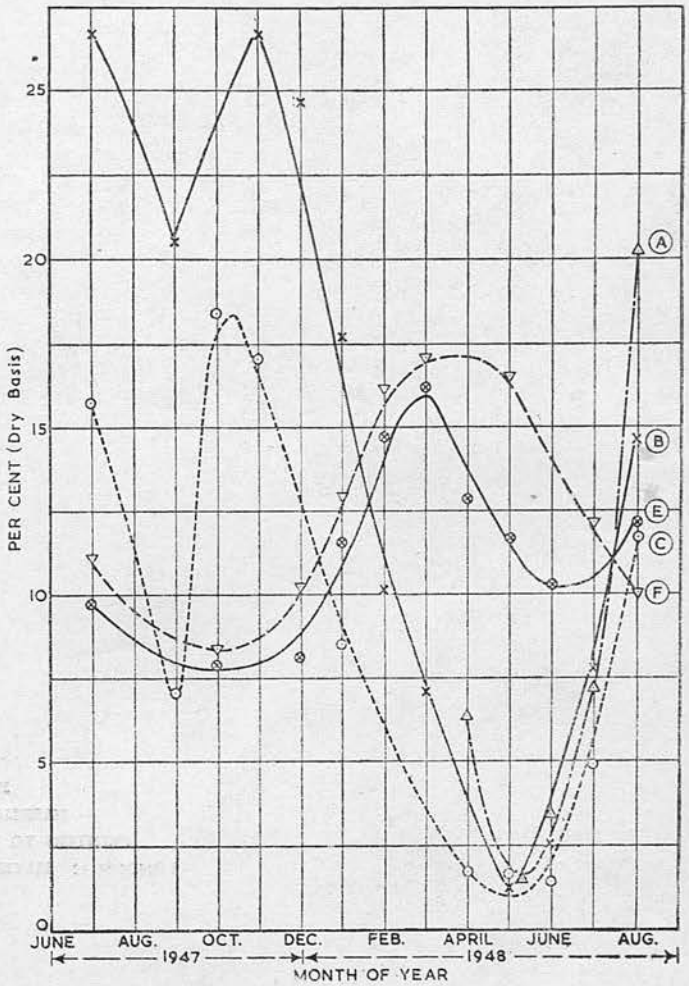

FIG. 8. *Seasonal variation in* L. cloustoni *fronds*

A Laminarin at 4 m. B Laminarin at 8 m.
C Laminarin at 12 m. E Alginic acid at 8 m.
F Alginic acid at 12 m.

the fronds with very little variation in the stipes. In general, in the fronds an increase in mannitol occurs with depth, reaching a maximum at 6–10 m., indicating that maximum photosynthesis probably occurs at that depth, in agreement with the observations of previous workers. Below 10 m. the mannitol decreases again. Laminarin decreases progressively with depth; it is at a maximum at 3–6 m. and is absent in samples from a depth below 16 m.

In early summer, when conditions do not appear to be favourable for the synthesis of laminarin, equilibrium exists between the mannitol and the ash, but when the laminarin content is high the ash remains relatively constant with depth and correlation exists between the mannitol and the laminarin. In some cases the alginic acid in the fronds also varies with depth when it is the inverse of the mannitol. In general, the iodine content increases with depth. Slight seasonal variations in composition are evident in the stipes, coinciding with similar variations in the fronds. It would appear that the most marked variations in composition with depth occur in the summer when the energy of light is at a maximum; light may therefore be the chief limiting or controlling factor.

Acknowledgments

The work described in this paper forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association. The author wishes to thank Miss B. Graham and Mr. W. J. Cornhill for assistance with the analytical work; he also wishes to thank the Association for permission to publish.

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Received June 3, 1949

References

1 Black, W. A. P., J. Soc. chem. Ind. 1948, **67**, 165
2 Black, W. A. P., *ibid.* 1949, **68**, 183
3 Haas, P., and Hill, T. G., *Ann. Bot. Lond.* 1933, **47**, 55
4 Russel-Wells, B., *Nature* 1932, **129**, 654
5 Gail, F. W., *Pubns. Puget Sound Biol. Station* 1918/20, **2**, 139
6 Moore, B., *J. chem. Soc.* 1921, **119**, 1555
7 Manning, W. M., Juday, C., and Wolf, M., *Trans. Wis. Acad. Sci.* 1938, **31**, 377
8 Schomer, H. A., and Juday, C., ibid. 1935, **29**, 173
9 Moore, B., Whiteley, E., and Webster, T. A., *Trans. Liverpool Biol. Soc.* 36th Annual Report, **1922**, 32
10 Tikhovskaya, Z. P., *C. R. Acad. Sci. U.R.S.S.* 1940, **29** (2), 120
11 Parke, M., *J. Mar. biol. Ass. U.K.* 1948, **27** (3), 651
12 Levring, T., Goteborgs. K. Vet.-Och. Vitt-Samh-Handl., Sjatte foljden. Ser. B. Bd. 5, No. 6, 1947
13 Atkins, W. R. G., *Botaniska Notiser* **1939**, 144
14 Colin and Ricard, R., *Bull. Soc. Chim. biol., Paris* 1931, **13**, 417
15 Lunde, G., *Z. angew. Chem.* 1937, **50**, 731
16 Walker, F. T., *Nature* 1948, **161**, 977

the weed was draped over racks and dried at a temperature of 25–40° for 48 hours, prior to despatch to Edinburgh, where on arrival it was dried for a further 24 hours at 20–30°, and ground in a Christy and Norris No. 8 Laboratory Mill fitted with a $\frac{1}{64}$-in. perforated plate screen.

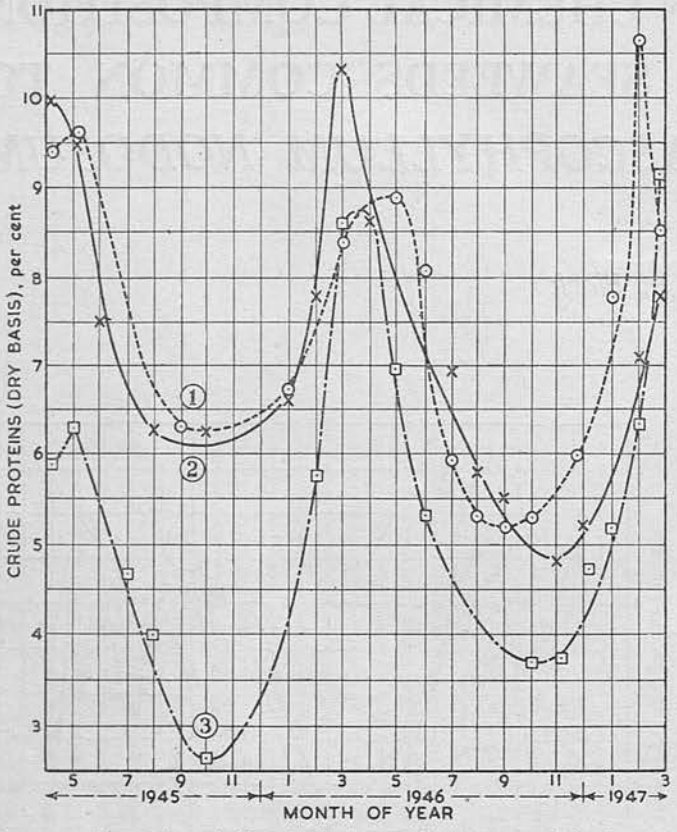

FIG. 3. *Seasonal variation in crude protein content.* (1) *Open sea.* (2) *Medium exposed.* (3) *Sea loch*

Experimental

The dry weight, total ash, iodine and crude protein contents were determined by the methods given in a previous communication,[1] and for mannitol, laminarin and alginic acid the methods reported by Cameron, Ross and Percival[10] were again used.

Discussion of results

General.—The results obtained are all calculated on the anhydrous basis and are summarized in the graphs (Figs. 1 to 6).

In the case of the laminaria (loc. cit.), the plants were divided into stipes and fronds, which were analysed separately. The results showed that the wide seasonal variations in chemical constitution are due almost entirely to variations in the fronds, which are annual, whereas the stipes are perennial, and it is in the fronds where most of the photosynthesis occurs. In the present investigation, the complete plant has been analysed and so we have been continually analysing old and new growth. This is, of course, true to a certain extent with the frond of the laminaria which is continually growing from the transition zone, and wearing away at the tip. Moss,[11] in studying the anatomical structure and chemical composition of *Fucus vesiculosus*, divided the plant into three sections, the distal, the middle and the proximal regions which she analysed separately and found wide variations in composition; thus the growing tips were always higher in mannitol and laminarin than the remainder of the plant. It is quite probable that a similar concentration gradient occurs in *Ascophyllum nodosum*. In addition to the concentration gradient along the thallus, the

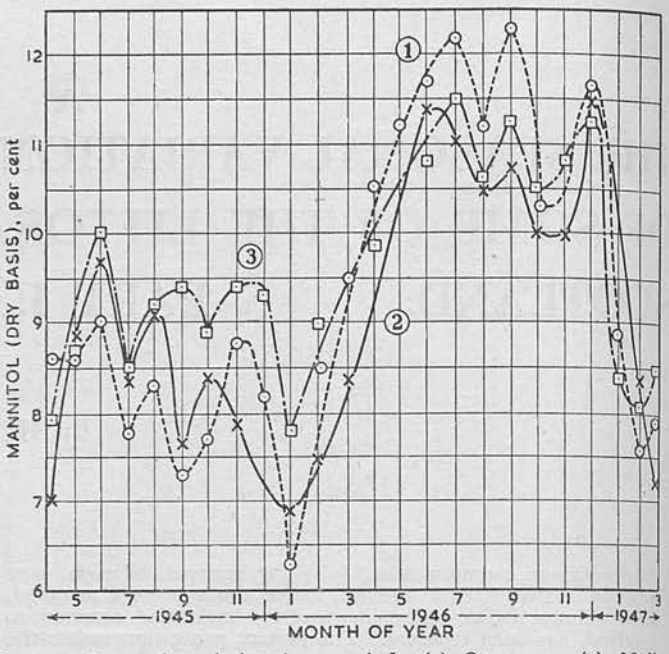

FIG. 4. *Seasonal variation in mannitol.* (1) *Open sea.* (2) *Medium exposed.* (3) *Sea loch*

complete plant when analysed does undergo a recognizable seasonal variation which bears some relationship to that of the laminariaceae, but within a much narrower range.

The most striking difference between the samples from the three habitats is in the crude protein content which in the loch samples is appreciably lower at the end of the year, no doubt due to the exhaustion of inorganic nitrates in the water, and the fact that they may not be so quickly regenerated as in the open sea.

In general, the mannitol, laminarin and dry weight contents of the loch samples are higher, and the ash, crude protein and alginic acid contents lower than in the open sea or medium exposed samples.

The most outstanding feature of the graphs, however, is that, in general, the laminarin, mannitol and percentage dry weight graphs show three distinct peaks in each year. It is interesting to note that this also occurs in a few of the samples of laminariaceae already examined, where maximum mannitol contents have been recorded in June, September and December. Seasonal variations, therefore, appear to have the same effect on the fucaceae as on the laminariaceae. The former, however, do not accumulate mannitol and laminarin to anything like the same

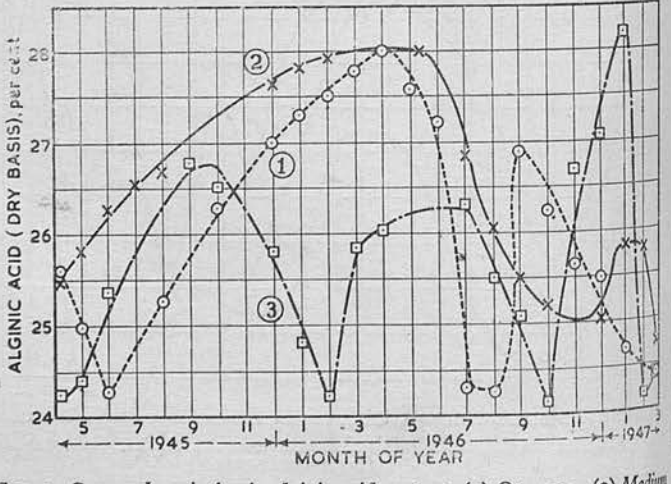

FIG. 5. *Seasonal variation in alginic acid content.* (1) *Open sea.* (2) *Medium exposed.* (3) *Sea loch*

extent as the latter, but throughout the year the alginic acid content is appreciably higher. This may be due to the physical structure of the fucaceae which may require a thicker cell wall to resist desiccation when the plant is exposed to long periods of hot sunshine, and alginic acid, which is exceedingly hygroscopic, with the power to resist desiccation, will be beneficial.

Total ash.—The ash graphs are approximately the inverse of the mannitol and laminarin graphs. Maximum ash contents occur from January to March, when the laminarin and mannitol are at a minimum. This is no doubt the time when photosynthesis is at a minimum and the plant is living on its reserves of mannitol, etc. The maximum ash content obtained was 26% as compared with over 50% in the annual *Sacchoriza bulbosa*. In the sea loch and open sea samples an increase occurs in July/August, followed by a drop in September. It is interesting to note that a drop in mannitol and increase in ash occurs also in July/August in the laminariaceae, and several explanations have been advanced, such as lack of nutrients in the sea at that time of the year.

Crude proteins.—The three samples give very similar graphs (Fig. 3) exhibiting maxima in January/February and minima October/November with the proteins appreciably lower in the loch samples.

Laminarin, mannitol, and dry weight contents.—The graphs for laminarin, mannitol and percentage dry weight contents bear a striking resemblance to each other, an increase in the mannitol and laminarin being accompanied by an increase in the dry weight content. With the open sea samples, e.g., in 1946, three distinct maxima in laminarin occur in June (4·3%), September (4·4%) and December (4·9%) coinciding with the maxima of the graphs for mannitol and percentage dry weight. In 1947, maxima again occur for laminarin in June (5·9%), September (6·1%) and November (5·9%). Similar peaks occur in the mannitol graphs and, as with laminarin, the mannitol is higher in the summer of 1947 than in the summer of 1946.

Alginic acid.—The alginic acid shows very little variation throughout the year, and, in the 72 samples analysed, only varied between 24 and 28%.

Iodine.—The iodine content varies between 0·07 and 0·20%. In general it is at a maximum from January to March and is lower in the loch samples than in the more exposed open sea samples.

Summary

The ash, iodine, crude protein, mannitol, laminarin and alginic acid contents of samples of *Ascophyllum nodosum* taken at monthly intervals from April, 1945, to March, 1947, have been determined. The samples were taken from three different habitats in the neighbourhood of Oban in order to show the effect of varying exposure.

In general, the loch samples are lower in iodine, total ash, crude proteins, and higher in laminarin and mannitol than the open sea or medium exposed samples. An increase in the mannitol and laminarin contents is accompanied by an increase in the dry weight content and a decrease in the total ash content. The graphs for the mannitol, laminarin and dry weight contents show three distinct peaks in the year, which agree with those in samples of the laminariacea already examined. The drop in mannitol and laminarin which occurs in the laminariaceae in July/August, and is at present the subject of investigation, also occurs in *Ascophyllum nodosum.*

Ascophyllum nodosum is appreciably lower in mannitol and laminarin than the laminariaceae, but laminarin is present throughout the year, whereas it is absent for periods in the laminariaceae so far investigated. This alga is higher in alginic acid than any of the laminariaceae examined. The very slight variations in mannitol, laminarin and alginic acid, throughout the year, enable this alga to be harvested for any particular constituent at any time of the year.

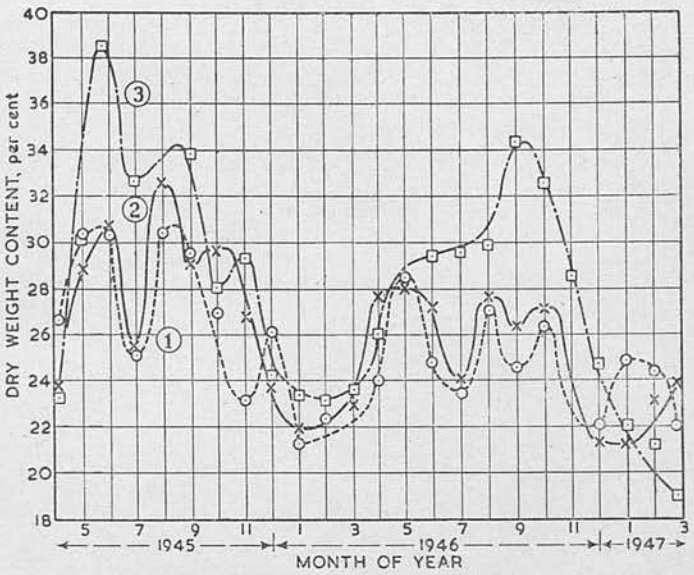

FIG. 6. *Seasonal variation in dry weight content.* (1) *Open sea.* (2) *Medium exposed.* (3) *Sea loch*

Acknowledgement

The work described in this report forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association, to whom the author is indebted for permission to publish.

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Received May 10, 1948

[1] Black, W. A. P., J.S.C.I. 1948, **67**, 165
[2] *Ibid.*, 169
[3] *Ibid.*, 172
[4] Hendrick, J., J. Board Agric. 1916, **22**, 1095
[5] Haas, P. and Hill, J. C., Biochem. J. 1929, **23**, 1000
[6] Colin, H. and Ricard, R., Compt. rend. 1930, **190**, 1514
[7] Lunde, G., Skand. Veterinarted Bakt., Patol Saint-Kolt-o-Mjolkhyg 1940, **30**, 26
[8] Vedrinskii, A. I., Trudy Arkhangel' skago Vodoroslevago Nanch-Isseldovatel. Inst. Voderosli Belago, Morga. 1938, 61
[9] Walker, F. T., Proc. Linnean Soc. 1947, **159**, 90
[10] Cameron, M. C., Ross, A. G. and Percival E. G. V., J.S.C.I. 1948, **67**, 161
[11] Moss, B., Ann. Bot. 1948, **12**, 267

*Reprinted from the Journal of the Society of Chemical Industry, 1949, **68**, pp. 183—189*

SEASONAL VARIATION IN CHEMICAL COMPOSITION OF SOME OF THE LITTORAL SEAWEEDS COMMON TO SCOTLAND.

PART II. *FUCUS SERRATUS, FUCUS VESICULOSUS, FUCUS SPIRALIS* AND *PELVETIA CANALICULATA*

By W. A. P. Black

In previous communications,[1, 2, 3] the seasonal variation over a period of two years in the ash, iodine, crude protein, mannitol, laminarin and alginic acid contents of the Laminariaceae common to Scotland, has been reported, while in a later paper,[4] the variations in the above constituents were given for the common littoral seaweed *Ascophyllum nodosum*. The work has now been extended to include the other common littoral algae, namely, *Fucus serratus*, *Fucus vesiculosus*, *Fucus spiralis* and *Pelvetia canaliculata* and the results have been correlated with the depth of immersion of these algae.

Fucus serratus, *Fucus vesiculosus*, *Fucus spiralis* and *Pelvetia canaliculata*, like *Ascophyllum nodosum*, belong to the littoral Fucaceae and are usually found growing in distinct zones at different water levels, *Fucus serratus*, *Fucus vesiculosus*, *Ascophyllum nodosum*, *Fucus spiralis* and *Pelvetia canaliculata* in that order from low to high water. At the highest level the fucoids are submerged only during high water at spring tides, and during long periods of exposure at neap tides they may experience wide changes in temperature and become quite brittle as a result of partial desiccation. Their existence is therefore aerial as well as aquatic.

Up to 1916 the amount of information available as to the composition of different seaweeds, and the variation in composition with age, season, depth of immersion, locality, etc., was extremely limited. During the 1914–1918 war the possibility of utilizing our seaweed resources as a source of potash was investigated by Hendrick[5] and *F. vesiculosus* and *F. serratus* were analysed for their inorganic constituents. In 1926 Pontillon[6] examined *F. serratus* and reported the presence of fucosan.

In 1930 Colin and Ricard[7] analysed samples of *F. serratus* and *Pelvetia canaliculata* and obtained the following results (%) on the dry basis.

	Date of harvest	Mannitol	Laminarin	Algin
F. Serratus	29.9.29	16·6	17·1	16·2
Pelvetia canaliculata	27.8.29	5·6	0	11·4

Haas and Hill[8] carried out the first systematic study of the metabolic products of the Fucaceae and examined various species for free reducing sugars. They concluded that there was present in *Pelvetia canaliculata* but not in *F. serratus*, a small amount of a free reducing sugar which was probably a pentose. The occurrence of mannitol was next investigated.[9] The isolation of crystalline specimens of mannitol from *F. serratus* by alcoholic extraction presented no difficulty but it was found impossible to obtain it from *Pelvetia canaliculata* and Haas and Hill showed that this was due to the presence of mannitan which occurred in a seaweed such as *Pelvetia canaliculata* which was exposed to long periods of drought and desiccation.

On investigating the nitrogen metabolism, Haas and Hill[10] found that aqueous extracts of certain brown algae gave a marked biuret reaction, the intensity of which diminished with the depth of immersion of the plant. They concluded that the material responsible was an octapeptide of glutamic acid which they isolated from *Pelvetia canaliculata*.

Continuing their study of the Fucaceae, Hass and Hill[11] found a direct decrease of fats and fat-like substances with depth of immersion, results of which were later confirmed by Russel-Wells.[12]

Lunde[13] investigating the possibilities of utilizing seaweed as fodder, analysed *F. serratus* and *F. vesiculosus* and obtained the following results (% dry basis).

	Alginic acid	Laminarin	Mannitol	Protein	Ash	Iodine
F. vesiculosus	19	2	5	9/18	22	0·05
F. serratus ..	25	4	6	9/18	22	0·07

Vedrinskii[14] after a study of the industrially important seaweeds of the White Sea concluded that *F. vesiculosus* and *F. serratus* were suitable for the production of alginic acid.

In 1941 Sosa and Sosa[15] at the time of the spring fructification of *F. vesiculosus* collected separately the male and female receptacles and analysed them for mannitol, proteins, fatty material and reducing sugars.

Although the products of metabolism of *F. vesiculosus* have also been studied by Kylin[16] and Butler[17] it was not until 1947 that anyone attempted to correlate its chemical composition with its anatomical structure. Moss[18] has shown that there is not only a correlation between anatomical structure and chemical composition but that plants of *F. vesiculosus* from three Scottish localities with different degrees of exposure to wave action, exhibited variations in external features, anatomical structure and chemical composition.

A review of the literature indicates, therefore, that no systematic work has been carried out on the seasonal variation in chemical composition of *F. serratus*, *F. vesiculosus*, *F. spiralis* and *Pelvetia canaliculata* and the present investigation is therefore unique in that respect.

A publication by Walker[19] gives the results of a recent survey of the Scottish coast and the quantities of the above algae which are available.

Preparation of samples

The samples were all taken at Aird's Point, thirty miles up the Firth of Lorne on the West Coast of Scotland, an area normally exposed to moderate wave action as it is protected from the Atlantic Ocean by the Islands of Lismore and Mull. At Aird's Point, *F. vesiculosus* attached to granite promontories of the mainland forms a well defined zone between *Ascophyllum nosodum* and *F. serratus* with a zone of *F. spiralis* and *Pelvetia canaliculata* occurring beyond the Ascophyllum.

The samples were collected, prepared for analysis and analysed as described previously.

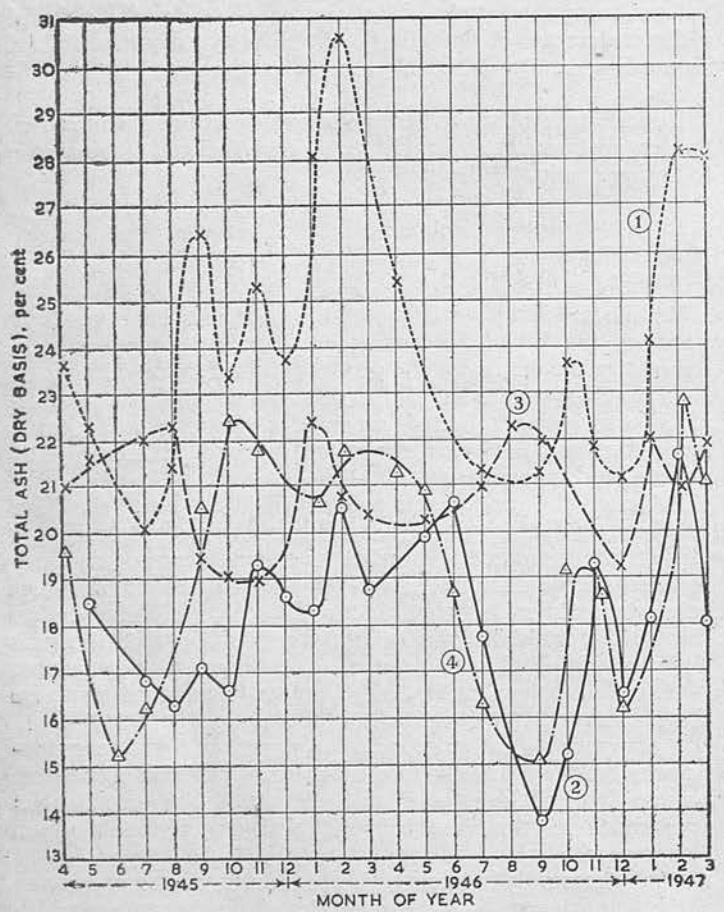
Discussion of results

General

The results on the anhydrous basis for the 24 months examined are summarized in Table I and in Graphs 1 to 8.

The results indicate that metabolism in the Fucaceae may differ somewhat from that in the Laminariaceae (*loc. cit.*). In the latter, the results expressed on the wet or dry basis show that the frond in the spring is high in ash and proteins and low in carbohydrates, but as photosynthesis proceeds, the percentage of carbohydrates increases and the percentage of ash decreases, and it appears to be a question of isotonic substitution, the cell sap as it enriches itself in carbohydrates becoming poorer in salts. In the Laminariaceae, laminarin, however, may play an important part in controlling the osmotic pressure of the cell sap, Moss (*loc. cit.*) states that such a simple explanation cannot be applied to the thallus of *F. vesiculosus*, and this appears to be true for *F. serratus*, *F. spiralis* and *Pelvetia canaliculata*.

It is not essential that algae should maintain a constant osmotic pressure, in fact Cooper and Pasha[20] have shown that the osmotic pressure in algae is always higher than that of seawater and undergoes a seasonal variation, increasing from October to May.



GRAPH 1. *Seasonal variation in Total Ash in—*

(1) *F. Serratus*
(2) *F. Vesiculosus*
(3) *F. Spiralis*
(4) *Pelvetia Canaliculata*

When the algae were exposed to air for long periods, as in the littoral zone, a considerable increase in the osmotic pressure occurred. No attempt, however, was made to correlate the chemical composition of the cell sap with the osmotic pressure.

On the other hand, Hurd[21] has shown that the osmotic pressure of *Nereocystis luetkeana* decreases as the salinity of the water is lowered and that on diluting the seawater with fresh water an increased intake of water results with an outward diffusion of salts from the cells.

When the results for the fucoids are expressed on the anhydrous basis as for *F. serratus* in Graph 7, the ash graph is roughly the inverse of the mannitol graph just as in the Laminariaceae. This is true also to a certain extent with the other fucoids although greater divergencies occur with increase in the depth of emergence. When the results, however, are expressed on the wet basis we have an entirely different picture and find as in Graph 8 that for *F. serratus* and *Pelvetia canaliculata* the ash and mannitol curves are approximately parallel, that is an increase in mannitol is, in general, not accompanied by a decrease in ash, and there appears to be no question of isotonic substitution.

The Fucaceae, however, differ from the Laminariaceae in that they lead an aerial and aquatic existence and on exposure may undergo partial desiccation. This desiccation may alter the concentration of the cell sap and increase the osmotic pressure, as found by Cooper and Pasha (*loc. cit.*). On submergence again the osmotic pressure will be reduced, by diffusion of water into the cells as observed by Hurd (*loc. cit.*). To get a true picture of the metabolism of the Fucaceae, therefore, it would be necessary to take the samples when submerged. It is not known to what extent this desiccation in the littoral zone interferes with photosynthesis and affects the chemical composition. Hygroscopic substances such as alginic acid and fucoidin are believed to minimize this desiccation on exposure and in addition the Fucaceae are considerably higher in fats and waxes than the Laminariaceae.

At the beginning of the year January-February the dry weight content is at a minimum corresponding with minimum mannitol, laminarin and ash contents. From February to June the ash and mannitol increase giving an increase in the dry weight content. With the Laminariaceae an increase in mannitol does not occur until April-May and it appears, therefore, that the rate of photosynthesis increases earlier in the year in the littoral zone.

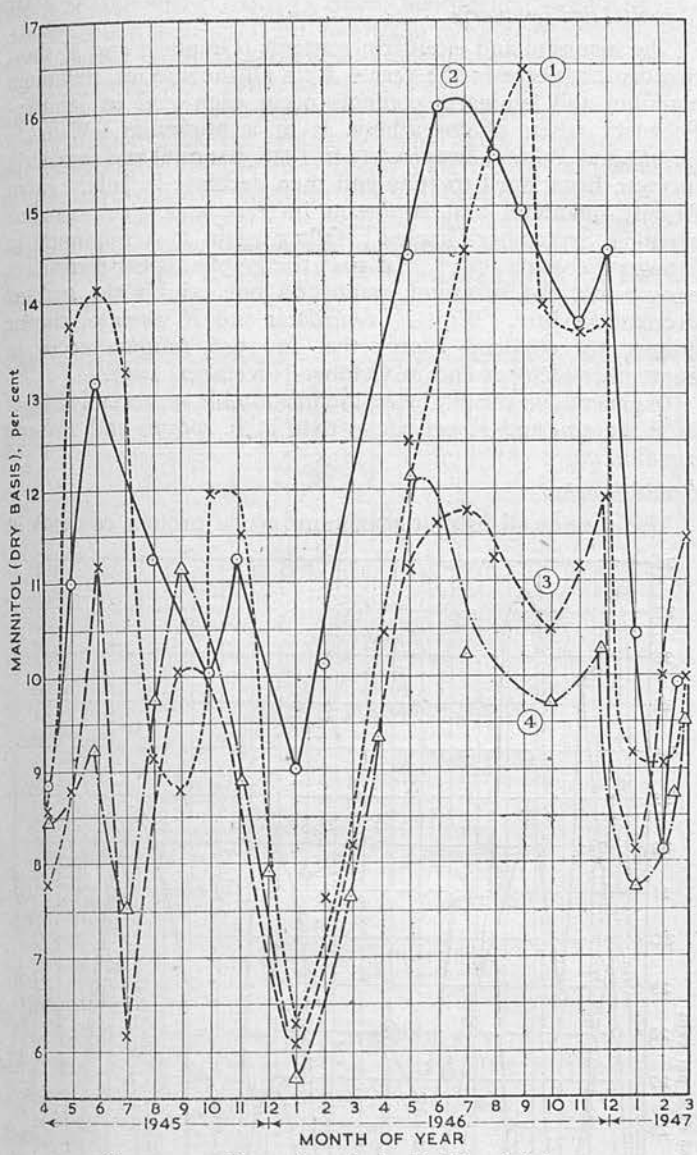
In July-August a considerable drop occurs in the dry weight contents accompanied in *F. spiralis* and *Pelvetia canaliculata* by a drop in laminarin and mannitol, on the anhydrous basis, while in *F. serratus* and *F. vesiculosus* the drop in laminarin and mannitol does not occur until September-October (Graphs 2 and 4). When the results are expressed on the wet basis (Graph 8), however, the drop in mannitol and laminarin contents coincides with the drop in the dry weight contents in July-August.

Moss (*loc. cit.*) in analysing the receptacles of the fertile thalli of *F. vesiculosus* found the dry weight content to be as low as 10% in July-August, and while the mature receptacles showed very low dry weight, low mannitol and low laminarin contents, the sterile tips of the same plant were increasing in mannitol and laminarin during that period. At this critical stage, the mature receptacles constituted more than half the total fresh

Table I

Maximum, minimum and average compositions

	Dry weight content			Total ash			Alginic acid			Mannitol			Laminarin			Crude proteins			Iodine		
	Max.	Min.	Ave.	Max.	Min.	Ave.	Max.	Min.	Ave.	Max.	Min.	Ave.	Max.	Min.	Ave.	Max.	Min.	Ave.	Max.	Min.	Ave.
Pelvetia canaliculata ..	37·5	21·2	28·1	22·9	15·2	19·4	18·6	13·3	15·3	12·3	5·8	9·3	5·2	1·8	3·5	12·1	4·5	7·8	0·06	0·03	0·04
Fucus spiralis	31·3	15·0	24·6	22·9	15·2	21·2	16·6	13·0	15·0	12·0	5·6	9·7	6·9	1·5	3·7	14·0	4·0	8·3	0·04	0·02	0·03
Fucus vesiculosus	35·7	19·0	27·3	20·7	13·8	18·3	17·2	13·8	15·5	16·1	8·3	12·1	4·9	2·0	3·5	10·9	5·9	7·9	0·06	0·03	0·05
Fucus serratus	33·4	19·1	24·9	30·5	19·6	24·0	22·1	17·0	19·6	16·5	6·3	11·3	10·2	2·1	4·6	14·9	6·4	9·7	0·10	0·05	0·08



GRAPH 2. *Seasonal variation in Mannitol in—*
(1) *F. Serratus*
(2) *F. Vesiculosus*
(3) *F. Spiralis*
(4) *Pelvetia Canaliculata*

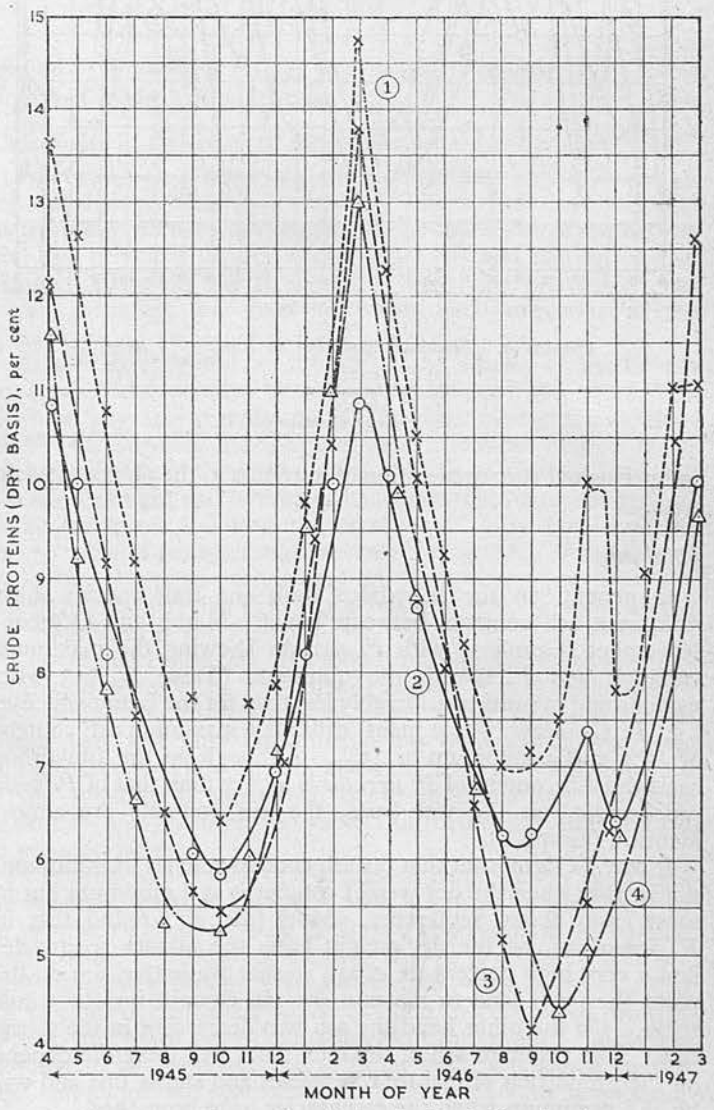
weight of the thalli and were, therefore, responsible for the low dry weight content of the whole plant. It is quite probable that in May-June, while the receptacles are maturing, a considerable amount of water is taken in to liberate the gametes, when there is considerable loss of intercellular mucilage. An analysis of the gametes and this mucilage would throw some light on this problem. It is unfortunate that in this investigation, an attempt was not made to correlate reproduction with chemical composition, and it is interesting to note the marked difference in chemical composition between the male and female receptacles of *F. vesiculosus*, as found by Moss and Sosa and Sosa (*loc. cit.*).

It is significant also that in the sub-littoral zone a break in the mannitol graphs and in some cases the laminarin graphs, has occurred in the three main Laminarias investigated. In this case, however, a marked drop in the dry weight content does not occur. Different theories have been advanced for this "supposed falling off" in photosynthesis. Light and reproduction may be contributory factors, Moore[22] having suggested that the light is too strong for photosynthesis at that time of the year. It is also significant, however, that this drop in mannitol should occur in the littoral and sub-littoral weeds in July-August when the seawater around our shores is denuded of nitrates and is extremely low in phosphates and the plants have used up their reserve of inorganic nitrogen which was present up to July (work in progress, to be reported later).

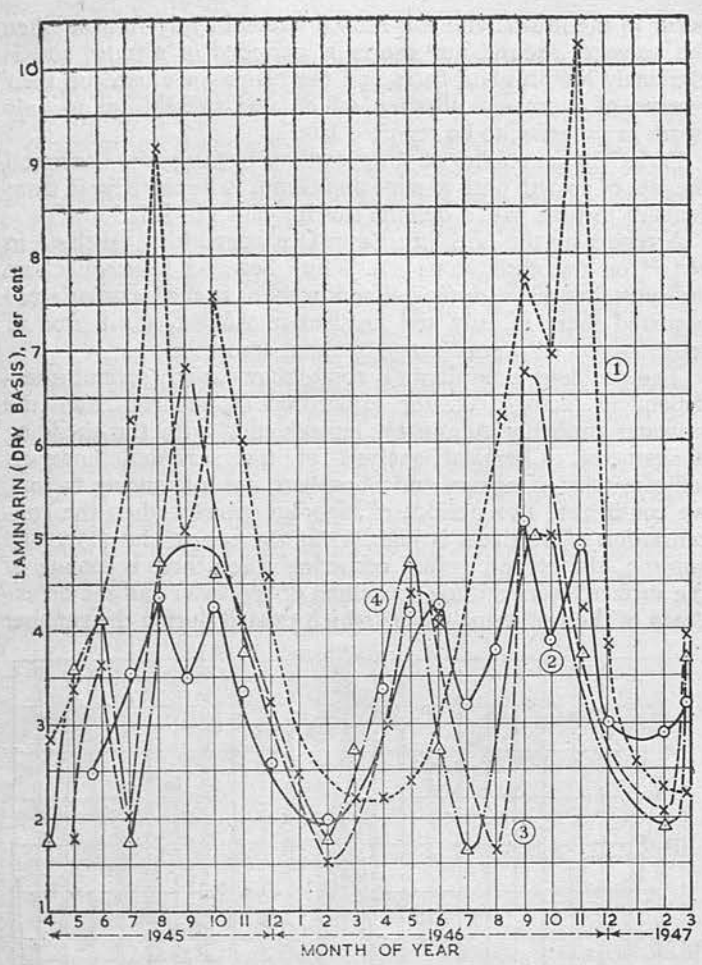
Parke[23] in her studies on the growth of *L. saccharina* has found the rate of growth both of stipe and frond, to be more rapid from January to June with a definite slowing down in July.

A report by the U.S.S.R. State Oceanographical Institute in 1931[24] on the algae along the White Sea and Murman Coast indicates that the beginning of sporogenesis in the Laminariaceae occurred there in July and finished in August, the period of reproduction showing a range of about six weeks.

The problem is no doubt a complex one, with several interdependent factors, e.g. the exhaustion of nutrients from the seawater probably culminates reproduction with the shedding of gametes. Chemical analysis of the seawater, however, indicates that if nitrate and phosphate are influencing factors, we could have two periods of vigorous growth when the concentration of nutrients is high (1) in the spring and (2) in the autumn, the second period occurring when there is cooling of the surface layers causing upwelling of the water and the breakdown of the isothermal layers which existed during the summer



GRAPH 3. *Seasonal variation in Crude Proteins in—*
(1) *F. Serratus*
(2) *F. Vesiculosus*
(3) *F. Spiralis*
(4) *Pelvetia Canaliculata*



GRAPH 4. *Seasonal variation in Laminarin in—*

(1) *F. Serratus*
(2) *F. Vesiculosus*
(3) *F. Spiralis*
(4) *Pelvetia Canaliculata*

and prevented the regeneration of nutrients in the photosynthetic layer.

Total ash

In general on the anhydrous basis the four species show maximum ash contents between January-March and October-November (Graph 1) with *F. serratus* showing the maximum variation and *F. spiralis* the minimum (Table I), but with maxima and minima considerably less than for the Laminariaceae, e.g., *L. Cloustoni* (whole plant) exhibits a maximum ash content of 43% and a minimum of 23%. Although on the anhydrous basis the ash content of *F. serratus* is higher than that of *Pelvetia canaliculata*, on the wet basis, the ash contents are almost identical (Graph 8).

It may be significant that the ash content reaches its minimum, not in July when the dry weight content is at a minimum but in most cases about September. Moss (*loc. cit.*) found that in *F. vesiculosus*, on the dry weight basis the mature receptacles had a very high percentage of ash so that while they are on the plant they will tend to increase the ash content for the whole thalli. On the other hand the ash was decreasing in the sterile tips. The resultant ash of the total plant will therefore depend on the proportion of mature receptacles and sterile tips and will be at a minimum when the receptacles have been shed.

The water insoluble ash contents have been found to vary between 4% and 6% (dry basis) which is of the same order as that found in the Laminariaceae.[1]

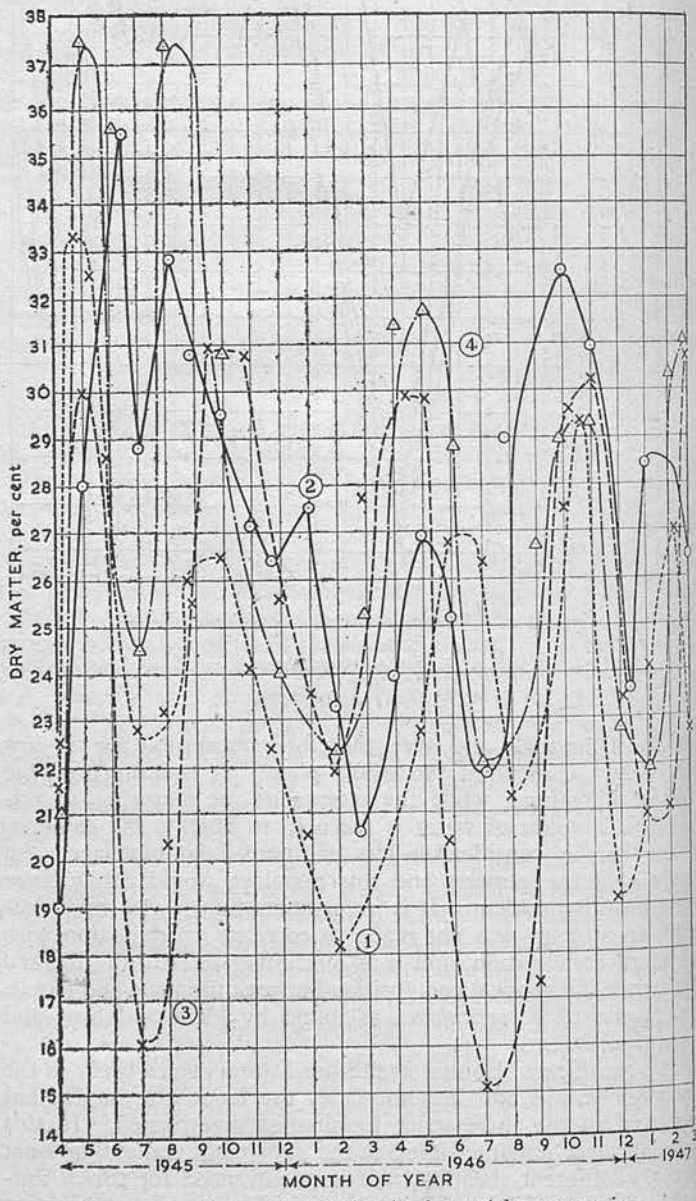
Mannitol and laminarin

The mannitol and laminarin contents (Graphs 2 and 4) show two distinct peaks in the year. With all the species, minimum mannitol and laminarin contents occur each year in January-February when photosynthesis is at a minimum. With *F. spiralis* and *Pelvetia canaliculata* in 1946 mannitol and laminarin increase from April to June and then decrease in July. From August, mannitol and laminarin increase and reach maxima again in September-October; falling again to a minimum in January-February, 1947. In 1947 the graphs repeat themselves except that the mannitol graphs do not exhibit the marked decrease in July. With *F. vesiculosus* and *F. serratus*, similar graphs are obtained except that in 1946 minima occur in September-October and in October-November, 1947.

In general, on the dry basis, the mannitol is appreciably higher in *F. serratus* and *F. vesiculosus* than in *F. spiralis* and *Pelvetia canaliculata*.

Crude proteins

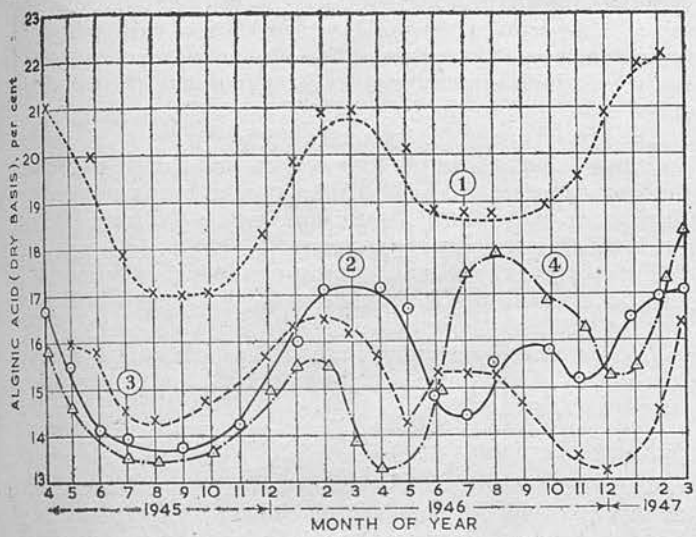
The species all exhibit maximum crude protein contents in



GRAPH 5. *Seasonal variation in dry weight content in—*

(1) *F. Serratus*
(2) *F. Vesiculosus*
(3) *F. Spiralis*
(4) *Pelvetia Canaliculata*

March of each year (Graph 3), when the inorganic nitrate in the seawater is at a maximum, and minimum in September-October, after a period of reproduction and at a time when the seawater has been completely denuded of nitrates. With the autumn mixing of the water layers and the regeneration of nitrates in the photosynthetic layer, the protein contents again increase. The only recognizable correlation between the crude protein content and the metabolism of the plant would appear to be that during the winter when nutrients are plentiful and photosynthesis is at a minimum the plant synthesizes proteins, a synthesis which can probably take place in the dark. From March until the end of the summer, the proteins are then utilized in growth and reproduction.



GRAPH 6. *Seasonal variation in Alginic Acid in—*

(1) *F. Serratus*
(2) *F. Vesiculosus*
(3) *F. Spiralis*
(4) *Pelvetia Canaliculata*

Iodine

The iodine content shows very little seasonal variation (Table I) being appreciably lower in all the species than in the Laminariaceae already examined (*loc. cit.*).

Alginic acid

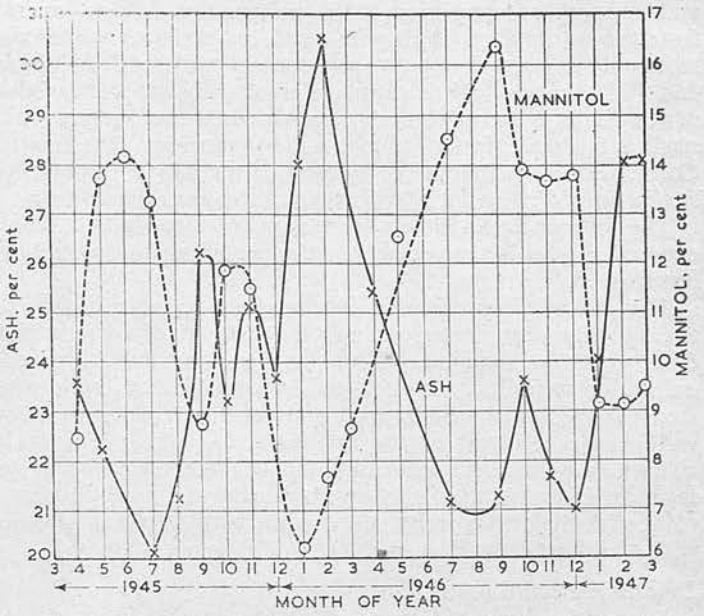
In general, on the anhydrous basis, the alginic acid is at a maximum from January-February and then decreases during the summer (Graph 6). This may not be a true decrease, however, as it is more than likely if the alginic acid is a cell wall constituent, that it will be a constant, the apparent decrease in summer being due to an increase in carbohydrates. A decrease may result in the summer however, if a stretching of the cell wall occurs, due to any increase in osmotic pressure.

Dry weight content

The four species show maximum dry weight contents in May-June of each year and again in September-November (Graph 5). In July-August of each year a considerable drop in dry weight occurs and an explanation has been advanced. It is noteworthy that *F. spiralis* contains the lowest dry weight content in July for, at that time, the mature receptacles are considerably swollen and contain large quantities of an aqueous mucilaginous substance.

It should be borne in mind, however, that there is no accurate method for the determination of dry weight contents. Fucoids on exposure undergo a certain amount of desiccation and the extent to which this occurs will depend on climatic conditions, and the period of time they have been exposed before the sample is taken.

The average dry weight contents over two years for the four species vary between 25 and 28% which is considerably greater than that of the Laminariaceae.



GRAPH 7. *Seasonal variation in Ash and Mannitol in* F. Serratus *on dry basis*

Correlation of the chemical composition with depth of immersion

Practically no knowledge exists regarding the relations of algae to their physical environment. The vertical and horizontal distribution will naturally depend on the conditions of the habitat and the principal factors affecting growth and distribution of algae are generally the temperature, light, composition of seawater, turbidity, movement of water, and interaction of the species.

Baker[25] investigated the causes of the zoning of the brown seaweeds and concluded that the time the weed was covered by the sea was the primary factor in determining the zoning. Gail[26] investigated the physical and chemical properties of seawater that might govern the distribution of algae and showed the effect of light and hydrogen-ion concentration on the growth of *F. evanescens* and how the presence of *Ulva lactuca* raised the p_H value of seawater and prevented the growth of the former even though other conditions of light were normal. His researches showed that light was a deciding factor in determining the lower limit of the Fucaceae.

Cooper and Pasha (*loc. cit.*) found that the hydrogen-ion concentration of seaweeds was one of the most important factors in determining the distribution of algae. The variation in the nitrogenous and fatty constituents has been shown by Haas, Hill and Russel-Wells (*loc. cit.*) and a variation in the composition of the sub-littoral algae with depth of immersion has been recorded[27] and has been the subject of further investigation (to be reported later).

In general, the results of the present investigation show that the seasonal variation increases with depth of immersion, i.e., we approach a plant of more constant composition as we emerge from the sea. This agrees with the change in anatomical structure, the algae growing in the lower zones, e.g., *F. serratus* and the Laminariaceae have become adapted to very rapid growth, and are subject to wide fluctuations in composition. With the Laminariaceae, however, the growing part is adjacent to the stipe and all growth takes place from this zone with a continual wear from the tips of the frond and in some cases a shedding of the old frond in the spring. With the Fucaceae, however, growth is from the tips, and Moss (*loc. cit.*) has found mannitol

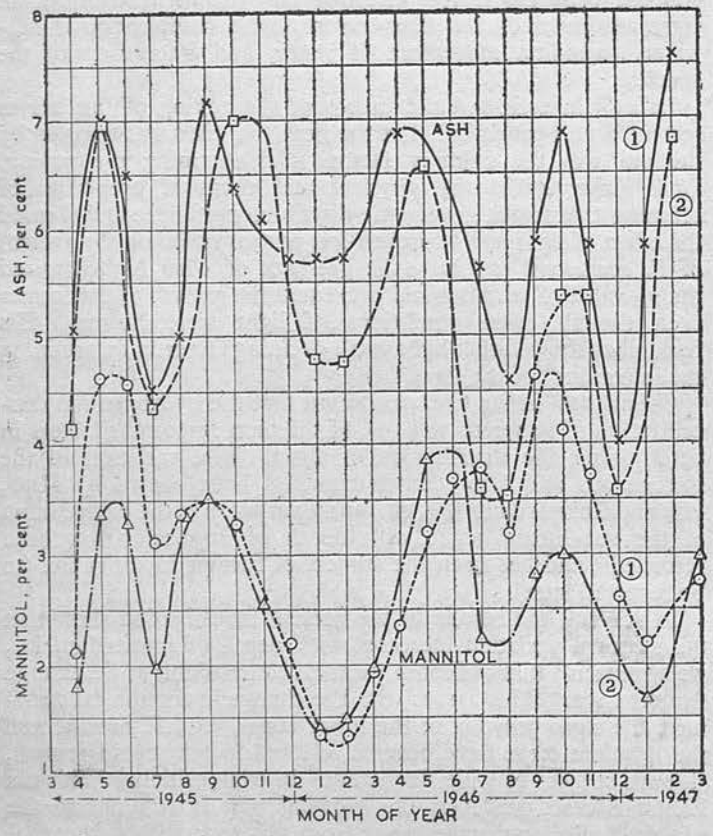
and laminarin at a maximum in the growing tips. As we emerge from the sea we have plants with a reduced surface/volume ratio more able to resist desiccation and consequently, with *F. spiralis* and *Pelvetia canaliculata* plants of more constant composition which do not require to store up such quantities of reserve foodstuffs as *F. serratus* which has to maintain a more vigorous growth. Consequently in the more exposed forms the percentage of laminarin and mannitol is lower than in the less exposed forms.

The difference in alginic acid content between the three most exposed species is very small but the percentage is appreciably less than in *F. serratus*.

The effect of reproduction on the chemical constitution has already been commented on and the method of reproduction differs with the zonation. With *F. spiralis* and *Pelvetia canaliculata*, for example, male and female receptacles are found with sterile fronds on the same plant and the effect on composition will be the resultant of the proportion of each present. In *F. serratus*, however, male and female receptacles occur on separate plants so that it is possible to have a sterile plant, a plant with male receptacles, and a plant with female receptacles with an influencing effect on the chemical composition.

Unidentified constituents

On averaging the analytical figures for the four species the ash, alginic acid, mannitol, laminarin and crude prot ins account for only about 60% of the dry matter while the same constituents account for approximately 85% of the dry matter of the Laminariaceae. The known constituents which remain to be determined are fats, pigments, cellulose and fucoidin and minor constituents like fucosterol and the algal terpenes which are at present being investigated. Russel-Wells has shown that the fats increase with depth of emergence from 0·16% in *L. digitata* to 3·6% in *Pelvetia canaliculata*. Work in progress indicates that fucoidin also increases with depth of emergence. The possibility of the presence of perhaps valuable constituents has yet to be explored.



GRAPH 8. *Seasonal variation in Ash and Mannitol in—*
(1) *F. Serratus*
(2) *Pelvetia Canaliculata*
on wet basis

Summary of results

The ash, iodine, crude protein, mannitol, laminarin and alginic acid contents of samples of *F. serratus*, *F. vesiculosus*, *F. spiralis* and *Pelvetia canaliculata* taken at monthly intervals from April, 1945, to March, 1947, have been determined.

The chemical composition has been correlated with the depth of immersion of the weed. In general, on the anhydrous basis, the ash, mannitol, laminarin and iodine contents decrease with depth of emergence. The Fucaceae show a very regular seasonal variation, the graphs for the dry weight, mannitol, laminarin and ash all show two maxima and two minima in each year. The figures indicate that the rate of photosynthesis increases earlier in the year in the littoral zone as compared with the sub-littoral zone.

The graphs giving the seasonal variation in the crude proteins are almost identical for the four species, and similar to those for the Laminariaceae. They do not appear to bear much relationship to the metabolism of the plant but can be correlated with the inorganic nitrate in the seawater.

The theory of isotonic substitution which applies to the Laminariaceae cannot be applied to the Fucaceae which appear to have a different metabolism.

The decrease in mannitol, laminarin, ash and dry weight content in July of each year is significant, and possible explanations have been advanced.

Acknowledgments

The work described in this report forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association, to whom the author is indebted for permission to publish. The author is also indebted to Miss Graham and Mr. Cornhill for assistance with the analytical work.

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Received January 20, 1949

[1] Black, W. A. P., J.S.C.I. 1948, **67**, 165
[2] *Ibid.*, 169
[3] *Ibid.*, 172
[4] *Ibid.*, 355
[5] Hendrick, J., J. of Board of Agric., 1916, **22**, 1095
[6] Pontillon, C., Comp. rend. soc. biol., 1926, **95**, 874
[7] Colin, H., and Ricard, R., Comp. rend., 1930, 1514
[8] Haas, P., and Hill, T. G., Biochem. J., 1929, **23**, 1001
[9] *Ibid.*, 1005
[10] *Ibid.*, 1931, **25**, 1472
[11] *Ibid.*, Ann. Bot., 1933, **47**, 55
[12] Russel-Wells, B., Nature, 1932, **129**, 654
[13] Lunde, G., Skand Veterinarted Bakt., Patol Saint Kolt-O-Mjolkhyg. 1940, **30**, 26
[14] Vedrinskii, A. I., Trudy Arkangel'Skago Vodoroslevago Nauch Isseldovatel' Inst. Voderosli Belago. Morga, 1938, **61**, 79
[15] Sosa, A., and Sosa, C., Bull. Lab. Maritime Dinard 1941, **23**, 43
[16] Kylin, H., Z. physiol. Chem. 1913, **83**, 17
[17] Butler, M., Plant Physiol., 1931, VI, 25
[18] Moss, B., Ann. Bot., 1948, **12**, 267 ; *ibid.*, Thesis for Ph.D. degree, London
[19] Walker, F. T., Proc. Linnean Soc., 1947, **159**, 90
[20] Cooper, R. E., and Pasha, S. A., J. Indian Bot. Soc., 1935, **14**, 237
[21] Hurd, A. M., Pubns. Puget Sound Biol. Station, 1918/20, **2**, 183
[22] Moore, B., J.C.S., 1921, **119**, 1555
[23] Parke, M., Jour. Mar. Biol. Assoc. U.K., 1948, **27**, No. 3, 651
[24] Transactions Oceanographical Institute, Moscow, 1931, Vol. 5, No. 3
[25] Baker, S. M., New Phytologist, 1909, 8, 196
[26] Gail, F. W., Pubns. Puget Sound Biol. Station, 1918–20, **2**, 139 and 287
[27] Black, W. A. P., Nature, 1948, **161**, 174

Printed by Harrison & Sons, Ltd. Printers to His Majesty the King. London: Hayes (Mx.): High Wycombe.

J. Mar. biol. Ass. U.K. (1954) 33, 49–60
Printed in Great Britain

CONCENTRATION GRADIENTS AND THEIR SIGNIFICANCE IN *LAMINARIA SACCHARINA* (L.) LAMOUR.

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(Text-figs. 1–5)

In previous publications the author (Black, 1948*a–c*, 1949, 1950*a*) has reported the seasonal variations in chemical composition that occur in certain of the common Phaeophyceae. In the sublittoral and littoral zones it has been found that a correlation exists between the chemical composition and the depth of immersion of the plant (Black, 1950*b*, and unpublished work). Differences in composition, particularly during the summer months, also occur in the same species taken at the same time and depth but from different habitats (Black, *loc. cit.*), and there is also evidence that in the laminarians the stage of development of the plant has a profound influence on the composition. In all the above-mentioned analyses the entire plants have been separated into stipe and frond, dried under controlled conditions, milled, and a well-mixed representative sample of each taken for analysis.

Work on the distribution of iodine and potassium in *Laminaria flexicaulis* (=*digitata*) has been carried out by Spindler (1948), Rinck (1948), Rinck & Brouardel (1949*a*, *b*), and Brouardel & Rinck (1950). Rinck & Brouardel (1949*a*) determined the iodine content of 325 samples of the fresh and dried alga, and found it to vary regularly with the distance along the frond, being at a minimum at the base of the frond where the youngest cells are and then increasing with the length of the frond. A similar variation was also obtained in the water content of different parts of the frond. As with iodine, they found a regular variation in the potassium content, the results indicating a direct relation between the morphological portions of the alga—holdfast, stipe, stipe-frondal zone and frond—and the concentration of the chemical elements. Moss (1948, 1950), in her studies on the structure and chemical composition of *Fucus vesiculosus*, has shown that a concentration gradient exists in the thallus and that the receptacles and sterile tips differ markedly in composition. As anatomical differentiation proceeded from the apex, the region of growth, to the base of the thallus, so did the chemical composition vary: the percentage dry weight and organic nitrogen (expressed as percentage fresh weight) increased while alginic acid decreased.

Recent work by Gerdes (1951) on the distribution of aneurin in the algae has shown a constant decrease in the aneurin content with distance from the

growing point. Sections of *Laminaria saccharina* were analysed from the transition zone, the upper part of the frond, the stipe and the holdfast. The transition zone contained 7·25 p.p.m. and the upper part of the frond 1·31 p.p.m. (dry basis).

Observations on the rate of growth of *L. digitata* and *L. saccharina* in the Dalne-Zelenetzky Gulf were made by Kuznetzov (1946), and Parke (1948) has shown that in *L. saccharina*, from the coasts of Devon and Argyll, there are two periods of growth, (i) a period of rapid growth from January to June/July with the most rapid growth between March/June, and (ii) a period of slow growth from July to December with the slowest period between September and December. Throughout a yearly cycle regular changes in growth rate affected the length, width, and thickness of the frond, while loss of frond tissue from the distal end started when a plant was a few months old and continued throughout its life. According to Parke (1948) there can be present in a *L. saccharina* frond in October a portion of newly formed tissue and tissue at least 7 months old. It is logical, therefore, to expect variations in composition with age. As the stipe of this species is relatively small compared to the frond, and as no variation existed in the dry-matter content along the stipe, only the frond was in each case analysed.

No work appears to have been carried out to show the presence of a concentration gradient in *Laminaria*. Moss (1948, 1950) has studied *Fucus vesiculosus*, but, whereas in *Fucus* the growing region is near the tips of the branches, in *Laminaria* it is at the base of the frond.

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Preparation of Samples and Methods of Analysis

For the investigation now to be reported samples were collected and treated as follows.

The initial sample of *L. saccharina* (L.) Lamour. was collected on 19 August 1949 at Shandon in the Gareloch at 4 m. (low water). This was plant 1, which had the dimensions shown in Fig. 1 A. The frond was divided transversely into five equal sections 30·5 cm. long and dried at 25–30° C. for 24 hr.

Plants 2–4 were collected in Kerrera Sound, Oban, Argyllshire, at approximately 4 m. (low water), on the 17 October 1950, 26 April 1951 and 30 May 1951 respectively, and sectioned as shown in Fig. 1. The sections were dried

at 100° C. for 16 hr. Each of the dried sections was then milled in a Christy and Norris no. 8 Laboratory Mill fitted with a 64-mesh screen, and analysed as previously described (Black, 1948*a*; Black & Cornhill, 1951).

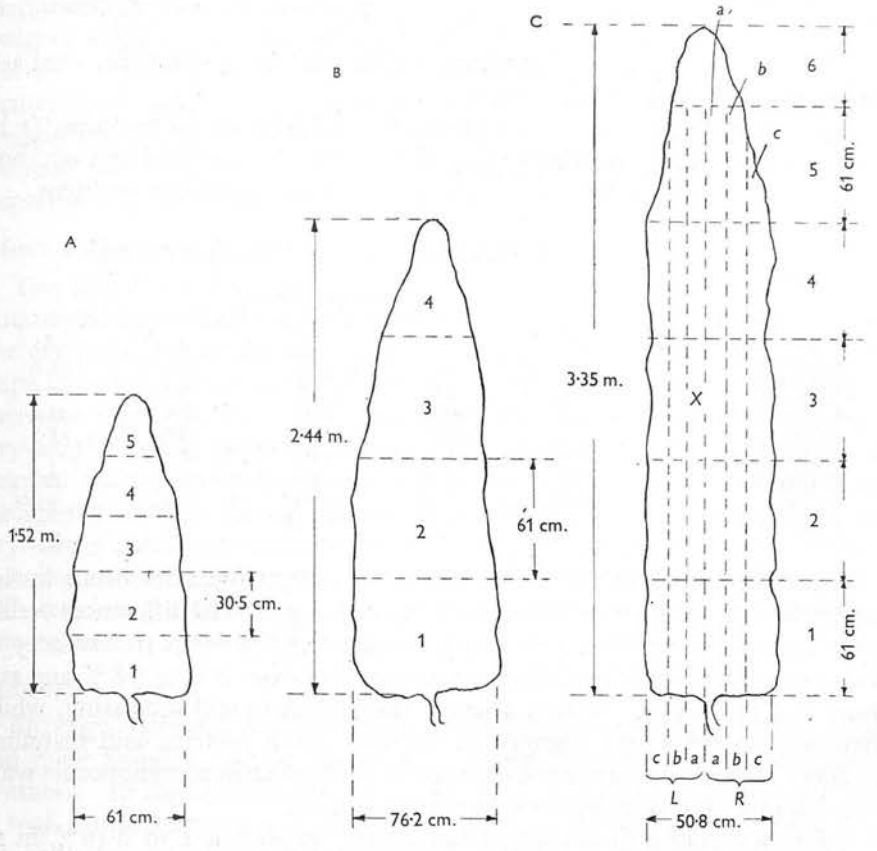

Fig. 1. Method of dividing the fronds investigated. A, plant 1, Gareloch, 19 August 1949. B, plants 3 and 4, Kerrera Sound, 26 April 1951 and 30 May 1951. C, plant 2, Kerrera Sound, 17 October 1950. All were divided into transverse 'sections' numbered from the base. In A there were five sections of 30·5 cm.; in B, four of 61 cm.; in C, six sections of which the first five were 61 cm. long and the terminal 30·5 cm. In C there were, in addition, longitudinal divisions. The frond was first divided into right and left halves (R and L). Each half was divided further into three strips, *a*, *b* and *c*, the first the innermost. $a=b=7{\cdot}6$ cm., $c=10{\cdot}2$ cm. (Any of the 30-odd subdivisions of the frond can be specified by a combination of three symbols: thus the unit marked *X* would be called L 3*a*.)

Results

The results obtained are expressed as fresh-weight and dry-weight percentages. When calculated as percentage concentrations in the water present the graphs were parallel to those for the fresh weight and have been omitted.

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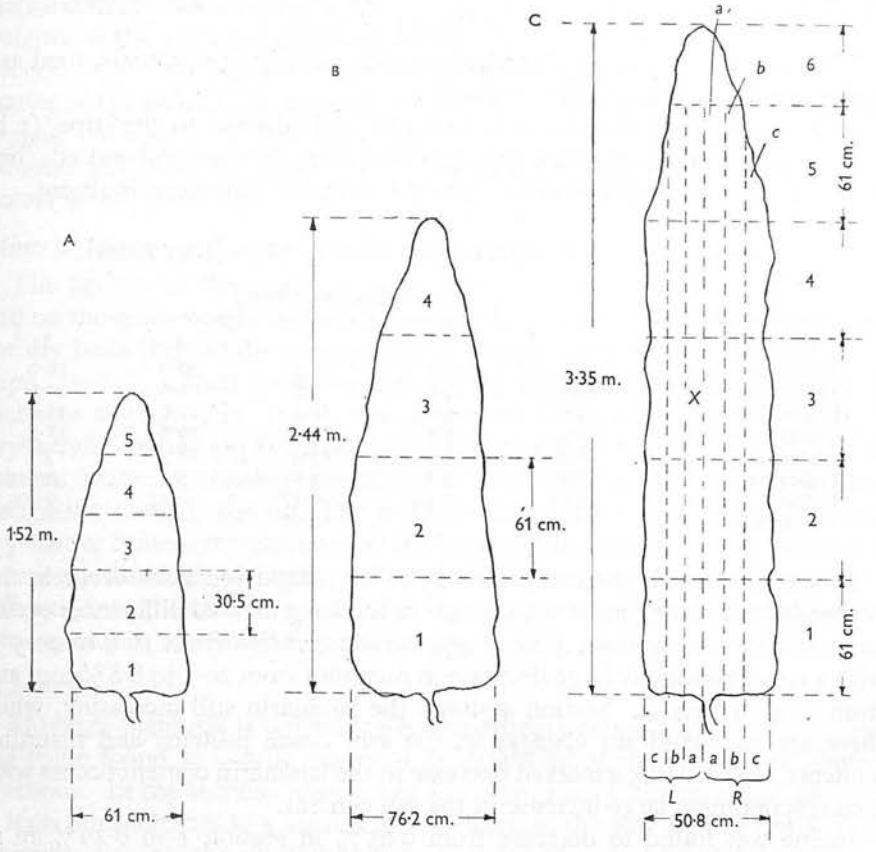

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Results

The results obtained are expressed as fresh-weight and dry-weight percentages. When calculated as percentage concentrations in the water present the graphs were parallel to those for the fresh weight and have been omitted.

The calculation of the results on the 'residual dry weight' was also considered, but, as this underwent considerable variation, calculation on the fresh weight, which approximately gives the results in terms of concentration in the water present, was preferred.

Plant 1

The figures for cellulose, laminarin, alginic acid, crude proteins, total ash and mannitol in plant 1 are given in Table I.

The results show that in the section of frond adjacent to the stipe, (1 in Fig. 1 A), which is the growing region of the plant, the mannitol and ash contents are high, the crude protein content is low, and laminarin is absent.

TABLE I. PLANT 1. PERCENTAGE COMPOSITION (DRY BASIS)

Percentage on dry basis	Section of frond 1	2	3	4	5
Total ash	28·8	26·4	19·4	20·0	26·0
Mannitol	29·5	30·6	8·8	9·6	10·6
Laminarin	1·0	4·0	16·4	22·0	12·4
Crude proteins	15·4	16·8	20·5	19·6	18·2
Alginic acid	8·4	13·4	13·4	18·3	17·7
Cellulose	3·8	3·8	3·8	3·8	3·8
Iodine	0·65	0·49	0·47	0·42	0·40
Fucosterol	0·10	0·06	0·06	—	—

In section 2, with the exception of the ash, slight increases occur in the content of the other constituents, while in section 3 marked differences occur, laminarin increasing from 4 to 16·4%, crude proteins from 16·8 to 20·5% with a correspondingly large decrease in mannitol from 20·5 to 8·8% and ash from 26·4 to 19·4%. Section 4 shows the laminarin still increasing, while there are no significant changes in the ash, crude proteins and mannitol contents. In section 5 a marked decrease in the laminarin content occurs with a correspondingly large increase in the ash content.

Iodine was found to decrease from 0·65% in section 1 to 0·49% in 2, 0·47% in 3, 0·42% in 4 and 0·40% in 5 (dry basis), while fucosterol decreased from 0·10% in section 1 to 0·06% in section 2, quite a significant difference for this sterol. The alginic acid content increased from 8% in section 1 to 18% in sections 4 and 5.

Plant 2

These results having shown that marked differences in composition occurred along the frond, a more detailed investigation was carried out with plant 2 which was divided into thirty-one areas (Fig. 1 C), one at the tip, with the five transverse sections subdivided each into six longitudinally. The frond being thickened medially the innermost sections were about twice as heavy per unit area as the outer with the percentage water only slightly higher in the central strip.

Dry Matter

As one passes up the axis of the frond there is a marked increase in dry matter (Fig. 2). This reaches a maximum two-thirds of the way up and then decreases towards the eroded tip. It is interesting that although the dry-matter content increases, e.g. from 12·6% in L 1*a* to 31·1% in L 4*a* the fresh weights of the sections remain practically constant (45·5 and 47·0 g respectively). Also sections L 1*a* and R 1*a* contain double the amount of dry matter of the adjoining sections L 1*b* and R 1*b*, but the same percentage dry weight, indicating that they have approximately the same percentage composition but differ in thickness. No significant difference in concentration occurs across the frond.

Mineral Matter

The figures for the mineral matter (total ash) expressed on the dry basis, and on the fresh-weight basis, are given in Figs. 2 and 3. When expressed on the dry basis (Fig. 2) the mineral matter is high in the sections adjoining the stipe (25–35%), then decreases half-way up the frond (19–23%), and then increases slightly. This is also true if the ash is calculated on the 'residual dry-weight' basis. On the fresh basis (Fig. 3) almost the reverse occurs, the mineral matter increasing two-thirds of the way up the frond and then decreasing towards the tip. The mineral matter, therefore, increases as the dry-matter content increases and as the mannitol decreases. Possibly mannitol is being utilized in respiration or for further synthesis and mineral matter is diffusing into the cells to control the osmotic pressure. The concentration of mineral matter across the frond is somewhat irregular.

Mannitol

Although mannitol is unlikely to be the primary product of photosynthesis the value found is usually regarded as indicative of the amount of photosynthesis. In the sections adjacent to the stipe (Fig. 3) the mannitol content is high but increases to a maximum one-third of the way up the frond then decreases towards the tip, falling from 35% to 11·5% of the dry matter.

Laminarin

Laminarin is regarded as a storage foodstuff. It is absent from the stipe of the laminarians and from the frond during the period of rapid growth in the spring. It appears to be formed during the period of slow growth when the nitrate and phosphate content of our inshore waters is very low. Laminarin is almost entirely absent from plants growing in habitats where the above nutrients are available all the year as a result of pollution or upwelling. The results for this carbohydrate are given in Fig. 4. Laminarin is entirely absent from the active growing sections adjacent to the stipe, and increases up the frond to 30·4% in L 4*c* and 34·5% in R 3*b*; in general it increases with the dry-matter content and then decreases to 5% at the tip.

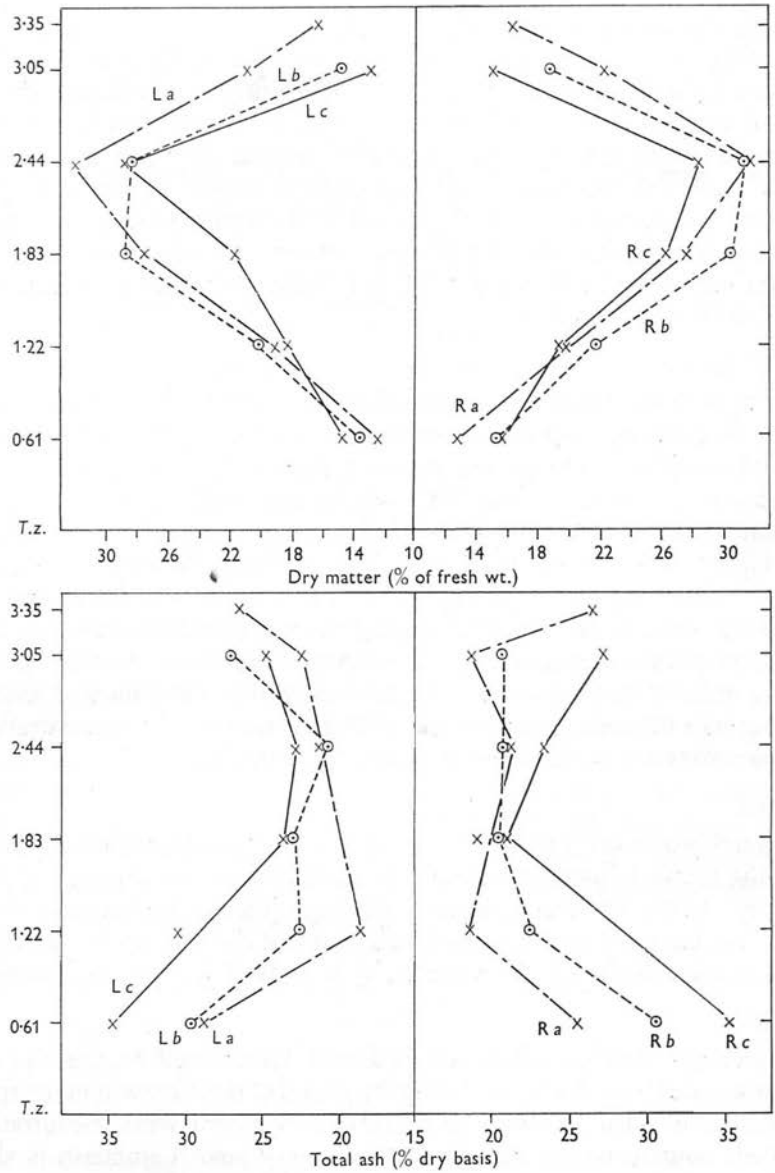

Fig. 2. Plant 2. Variation in (above) *dry-matter content* as percentage of fresh weight, and (below) *total ash* as percentage of dry matter. Each graph line represents one strip of the frond, with one value entered for each section. Left and right sides are graphed separately. *T.z.*, transitional zone at base of frond. Vertical scale *d* represents distance along the frond, from the base, in metres.

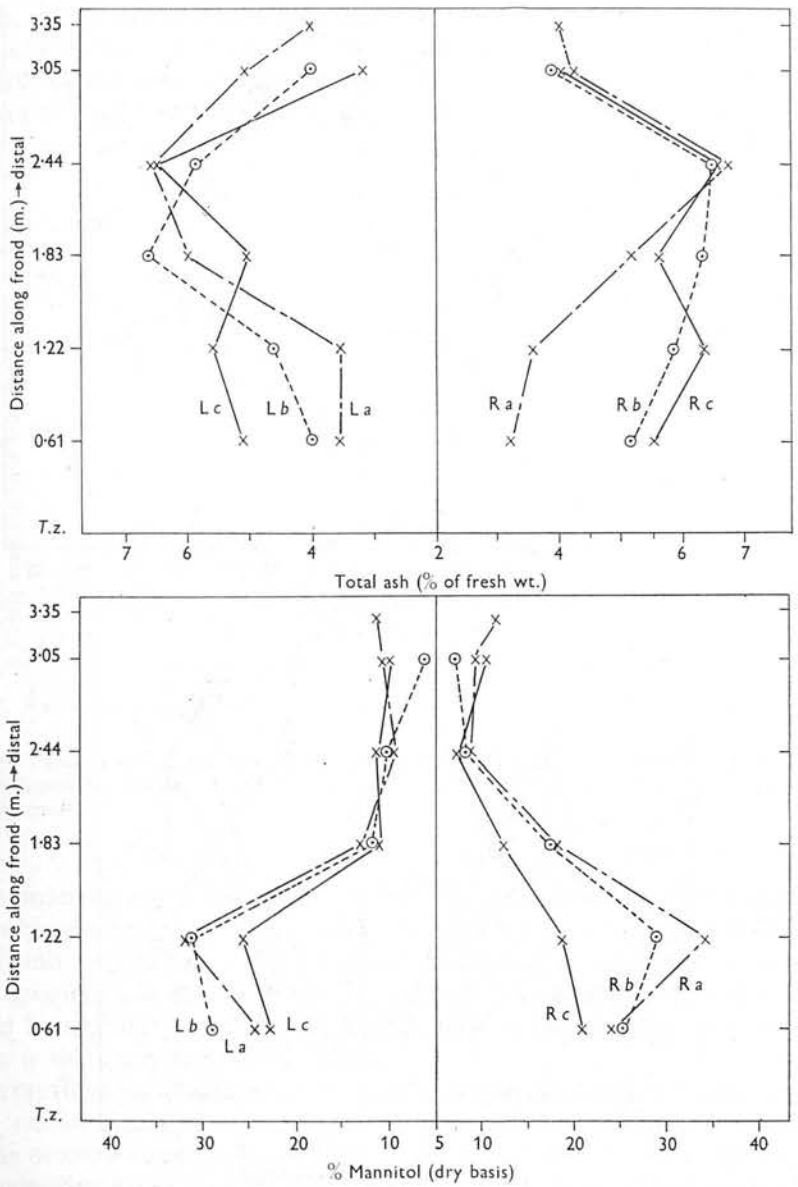

Fig. 3. Plant 2. Variation in (above) *total ash* as percentage of fresh weight, and (below) *mannitol* as percentage of dry matter. Particulars as in Fig. 2.

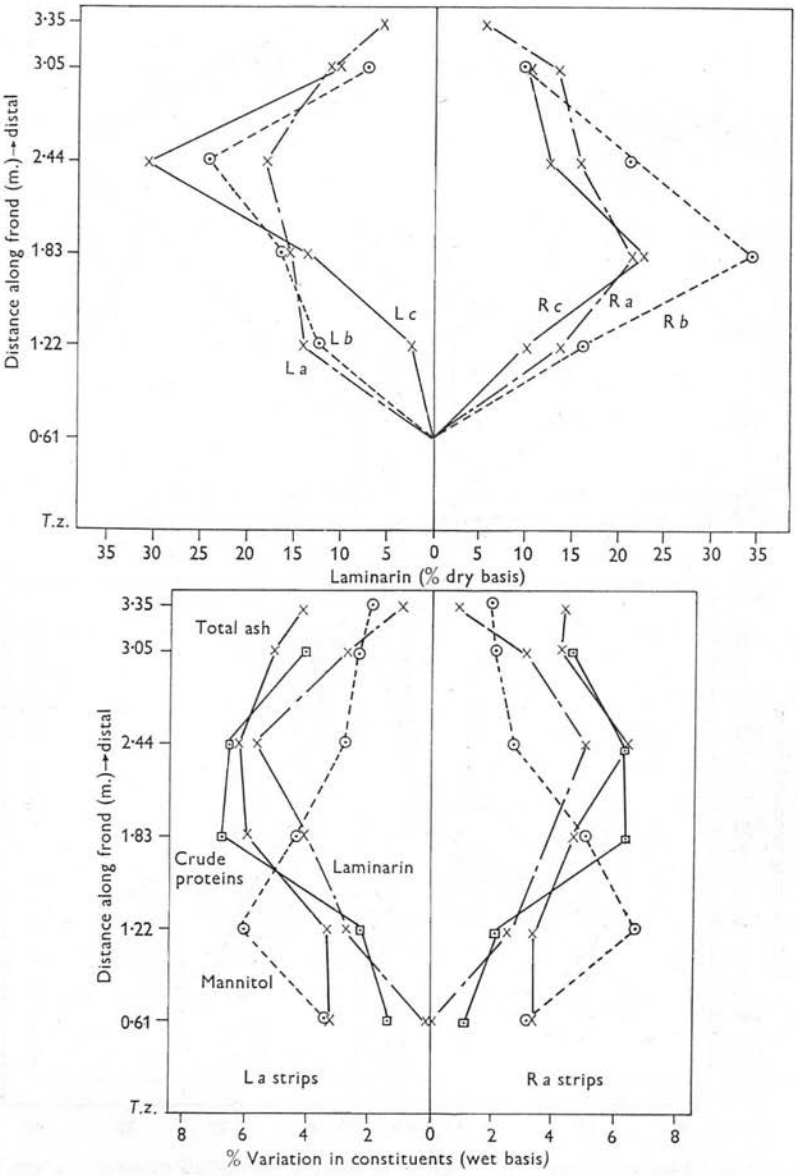

Fig. 4. Plant 2. Above: variation in *laminarin* as percentage of dry matter. Below: percentage variation in four constituents compared, on a wet basis, only the *a* strips considered.

Alginic Acid

The alginic acid content is exceedingly low in the sections L 1*a* and L 2*a* and is of such a low grade (i.e. of low molecular weight) that it is indeterminable by the standard method of analysis. In L 3*a* and L 4*a* values of 13·6 and 13·1% were obtained, indicating that a higher polymer of alginic acid is no doubt present.

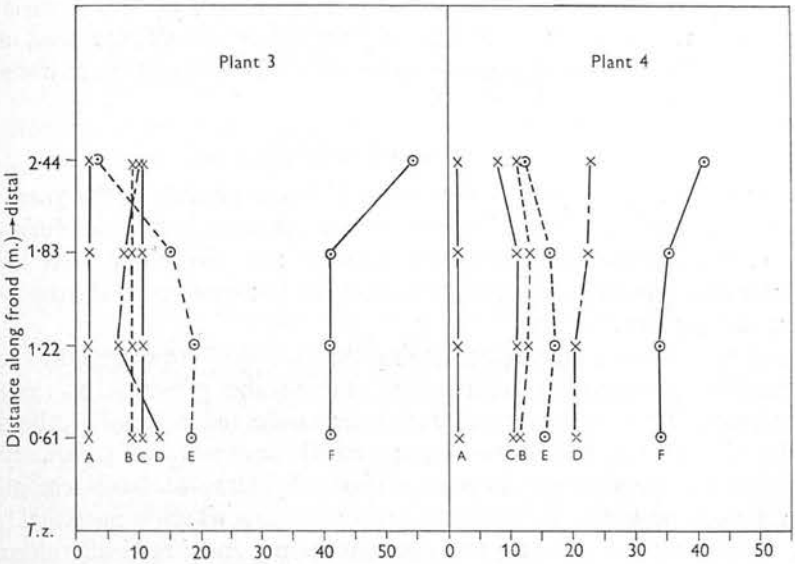

Fig. 5. Plants 3 and 4, illustrating the period of rapid growth. Variation in (A) *laminarin*, (C) *crude protein*, (D) *alginic acid*, (E) *mannitol*, (F) *total ash* (on dry basis); and (B) *dry matter* (on wet basis)

Plants 3 *and* 4

In order to prove that variations such as those recorded above were improbable during the period of rapid growth in the spring, plants 3 and 4 were taken and sectioned as in Fig. 1 B, and the analytical results are given in Fig. 5. The graphs show clearly that at this time of the year the composition of the frond is constant throughout its length, only at the tip where erosion takes place is there any noticeable change.

In previous publications (Black, 1948*b*, 1950*a*) the composition of the frond of *L. saccharina* is given for a period of 4 years, and the results agree with those of the present investigation. Plants 3 and 4, taken in April and May during the period of rapid growth, are composed mainly of tissue laid down during the preceding 3 months with perhaps only the distal part retained from the previous year, whereas plants 1 and 2 are composed of new tissue adjacent to the stipe and tissue up to 7–8 months old.

Analysis of a frond of *L. cloustoni* in May, when it was possible to differentiate between the new and old growth, showed that the old frond was low in

mannitol but still contained laminarin, while the new frond was appreciably higher in mannitol but contained no laminarin. In April/May the composition of an *L. saccharina* frond will, therefore, depend on whether old growth from the previous year has been retained or cast. From May onwards loss of frond is then due to erosion at the tip.

In Fig. 4 the graphs for the sections representing the central portion (L*a* and R*a*) have been superimposed and give a general picture of the variations in composition up the frond. In this case the results are all expressed on the wet basis, which is believed to give a more accurate picture of the living plant.

Discussion of Results

Four fronds of *L. saccharina*, collected at different periods of the year, have been sectioned and analysed. The two fronds taken in April/May during the period of rapid growth are composed chiefly of new tissue and there is little difference in composition along the length of the frond except at the tips where erosion has occurred.

The plant collected in August, during the period of slow growth, showed considerable variations in composition due to the presence of tissue of different ages. This was confirmed in the plant collected in October, the frond of which was cut into thirty-one sections which were weighed separately and later dried and analysed for dry matter, total ash, mannitol, laminarin, alginic acid and crude proteins. In the active growing region which is proximal to the stipe, where cells are dividing to form new tissue, and practically along the whole length of the frond during the period of rapid elongation, laminarin is absent and the dry-matter content is very low (10–12%) and is composed chiefly of mineral matter (36–40%) and mannitol (20–30%). In the active growing region the proteins are at a minimum, and the alginic acid is of such a low grade as to be indeterminable by the standard method. The results are in good agreement with those of Moss (1948) who found the dry matter and inorganic nitrogen, for example, lowest, and the mineral matter highest in the distal samples (tips) of *Fucus vesiculosus* where the growth is apical.

During the period of rapid growth there is extensive elongation due to cell enlargement with vacuolation. Then the tissue is low in dry matter composed mainly of mannitol and mineral matter and laminarin is absent. In July (Parke, 1948) a period of slow growth commences with the slowest growth between September and December when plants 1 and 2 were collected. June to September is also the period (Black & Dewar, 1949) when the nitrate and phosphate content of our inshore waters is at a minimum. During the period of slow growth, therefore, the stipo-frondal zone would alone represent new cells while the remainder of the frond, although able to photosynthesize, ceases active cell division and accumulates carbohydrates fated to become the food reserve for the winter or for sporogenesis. What Parke described as

mature tissue is, therefore, the storage portion of the frond two-thirds of the way up and containing a reserve of laminarin with proteins; the distal portions represent tissue which has spored or is badly eroded. Iodine and fucosterol are highest in the stipo-frondal (or transition) zone where they are in all probability required for new growth.

The results show that at certain times of the year concentration gradients of carbohydrates, proteins and mineral matter exist up the frond; they may be the result of restricted growth and may not be present in plants from habitats where nutrients are available all the year round and where laminarin has already been shown to be absent (Black, 1950*a*).

There is no evidence of a concentration gradient across the frond although the thickness shows considerable variation.

REFERENCES

BLACK, W. A. P., 1948*a*. Seasonal variation in chemical constitution of some common British Laminariales. *Nature, Lond.*, Vol. 161, p. 174.

—— 1948*b*. The seasonal variation in chemical constitution of some of the sublittoral seaweeds common to Scotland. Part I. *Laminaria cloustoni*. Part II. *Laminaria digitata*. Part III. *Laminaria saccharina* and *Saccorhiza bulbosa*. *J. Soc. chem. Ind., Lond.*, Vol. 67, pp. 165–8, 169–72, 172–6.

—— 1948*c*. Seasonal variation in chemical composition of some of the littoral seaweeds common to Scotland. Part I. *Ascophyllum nodosum*. *J. Soc. chem. Ind., Lond.*, Vol. 67, pp. 355–7.

—— 1949. Seasonal variation in chemical composition of some of the littoral seaweeds common to Scotland. Part II. *Fucus serratus*, *F. vesiculosus*, *F. spiralis*, and *Pelvetia canaliculata*. *J. Soc. chem. Ind., Lond.*, Vol. 68, pp. 183–9.

—— 1950*a*. The seasonal variation in weight and chemical composition of the common British Laminariaceae. *J. Mar. biol. Ass. U.K.*, Vol. 29, pp. 45–72.

—— 1950*b*. The effect of the depth of immersion on the chemical constitution of some of the sublittoral seaweeds common to Scotland. *J. Soc. chem. Ind., Lond.*, Vol. 69, pp. 161–5.

BLACK, W. A. P. & CORNHILL, W. J., 1951. A method for the estimation of fucosterol. *J. Sci. Fd Agric.*, Vol. 2, pp. 387–90.

BLACK, W. A. P. & DEWAR, E. T., 1949. Correlation of some of the physical and chemical properties of the sea with the chemical constitution of the algae. *J. Mar. biol. Ass. U.K.*, Vol. 27, pp. 673–99.

BROUARDEL, J. & RINCK, E., 1950. Potassium in *Laminaria flexicaulis* and the supposed distribution of this element. *Bull. Inst. océanogr. Monaco*, No. 967, 30 pp.

GERDES, G., 1951. Aenderungen in Aneuringehalt von Algen. *Arch. Mikrobiol.*, Bd. 16, No. 1, pp. 53–77.

KUZNETZOV, V. V., 1946. About certain peculiar features in the ecology and growth of *Laminaria digitata* (L.) Lamour. *C.R. Acad. Sci. U.R.S.S.*, Vol. 54, No. 6, pp. 533–6.

MOSS, B. L., 1948. Studies in the genus *Fucus*. I. On the structure and chemical composition of *F. vesiculosus* from three Scottish habitats. *Ann. Bot., Lond.*, Vol. 12, pp. 267–79.

MOSS, B. L. 1950. Studies in the genus *Fucus*. II. The anatomical structure and chemical composition of receptacles of *F. vesiculosus* from three contrasting habitats. *Ann. Bot., Lond.*, Vol. 14, pp. 395–410.

PARKE, M., 1948. Studies on British Laminariaceae. I. Growth in *Laminaria saccharina* (L.) Lamour. *J. Mar. biol. Ass. U.K.*, Vol. 27, pp. 651–709.

RINCK, E., 1948. Iodine in *L. flexicaulis*. *Bull. Lab. marit. Dinard*, T. 30, pp. 40–2.

RINCK, E. & BROUARDEL, J., 1949*a*. Iodine in *L. flexicaulis* and the supposed transmutation of this element. *Bull. Inst. océanogr. Monaco*, No. 959, 48 pp.

—— —— 1949*b*. Iodine in *L. flexicaulis*. *C.R. Acad. Sci., Paris*, T. 228, pp. 263–5.

SPINDLER, H., 1948. Potassium in *L. flexicaulis*. *Bull. Lab. marit. Dinard*, T. 31, pp. 1–8.

Reprinted from the Journal of Applied Chemistry, 1951, 1, *pp.* 414–424

MANUFACTURE OF ALGAL CHEMICALS. II. LABORATORY-SCALE ISOLATION OF MANNITOL FROM BROWN MARINE ALGAE

By W. A. P. BLACK, E. T. DEWAR and F. N. WOODWARD

Methods for the isolation of mannitol from the common brown algae indigenous to Great Britain have been worked out on the laboratory scale with a view to the ultimate development of a process suitable for large-scale production. These comprise (i) solvent extraction of the dried seaweed and (ii) dilute-acid extraction of the fresh or dried seaweed, followed by evaporation of the extract to dryness and the isolation of mannitol from this extract by (*a*) solvent extraction (*b*) precipitation as a water-insoluble derivative or (*c*) the use of ion-exchange resins.

Introduction

D-Mannitol, m.p. 166° C., b.p. 295° C./3·5 mm., $[\alpha]_D$ − 0·49° (water), occurs widely in nature, having been detected in the tubers, roots and leaves of many plants, and is a major constituent of some types of manna (Tanret, 1902) and the brown algae (Black, 1948).

Although mannitol does not appear to have been produced commercially from the marine algae, the work of Skroznikova (1939, 1941) has indicated that it can be extracted with water, alcohol, pyridine and aqueous calcium chloride. The process patented by Berk (1942), which has now lapsed, depends upon methanol or ethanol extraction.

The methods investigated, with the exception of the first, which is direct solvent-extraction, are all based on preliminary treatment of the seaweed with dilute mineral acid as this is the first stage in the production of alginates (Bashford, Thomas & Woodward, 1950) and laminarin (not yet published), and obviates the difficulties caused by case hardening and thermal degradation encountered when hot organic solvents are used. The aqueous acid extract after neutralization and evaporation to dryness affords a mixture of salts, mannitol and soluble polysaccharides, the separation of mannitol from which has been attempted by four different methods : direct extraction with methanol ; precipitation of laminarin by addition of ethanol to the aqueous solution of the mixture followed by (*a*) extraction of mannitol from the residue, with methanol, ethanol, *n*-butanol, *n*-propanol, pyridine or concentrated hydrochloric acid or (*b*) precipitation of mannitol as an insoluble derivative or (*c*) separation of mannitol and salts using ion-exchange resins.

The possible removal of mannitol from fresh algae by mechanical expression of the cell sap will be the subject of a future investigation.

Species examined

The species examined with their composition (dry basis) are given in Table I. After collection the samples were dried on a rack at a temperature of 25–30° C. for 48 hours and ground in a Christy and Norris mill fitted, except in the case of *L. cloustoni* when a 64-mesh screen was used, with a 32-mesh screen. For one experiment the *L. digitata* was remilled using a 64-mesh screen.

Table I

Composition of species examined

Nos.	Species	Habitat	Date Collected	Chemical composition (dry basis), % Ash	Mannitol	Laminarin	Alginic acid	Organic nitrogen
1	*L. digitata* frond	Loch Melfort	11.6.45	19·3	25·5	14·0	19·3	1·01
2	*L. digitata* frond	Loch Melfort	16.10.45	19·3	17·8	23·8	19·3	0·68
3	*L. cloustoni* frond	Cullipool	12.10.48	20·3	15·4	29·2	10·0	1·70
4	*Ascophyllum nodosum*	Cullipool	10.7.46	16·1	9·1	4·2	24·2	0·95
5	*Pelvetia canaliculata*	Port Appin	1.5.46	20·8	12·3	4·7	13·8	1·43

1. Solvent extraction of dried seaweed

Experimental and results

Two samples were used in these experiments, *L. digitata* frond and *L. cloustoni* frond, nos. 1 and 3 in Table I. Except where otherwise stated the dried milled weed was rendered anhydrous by heating in an oven at 105° C. for 16 hours.

The methanol and ethanol used were of commercial quality, and the *n*-propanol, *n*-butanol and pyridine were of the pure laboratory grade. The weed was extracted with the boiling solvent, either in a 1-l. round-bottom flask fitted with a Quickfit paddle-stirrer at 500 r.p.m. and reflux condenser, or in a Haanen and Badum apparatus. In the latter extractor the ground weed was placed in a 1 × 1 (Gallenkamp) sintered-glass crucible and continuously extracted with the

boiling solvent. The weed residue was then washed with ethanol and ether, dried at 100° C. for 45 min., weighed and analysed. The solution containing the extracted material was placed in the refrigerator for 2 days, and the mannitol which crystallized out was filtered and weighed. The methods of analysis were those previously used by one of the authors (Black, 1948). The results obtained are recorded in Tables II and III.

Table II

Solvent extraction of mannitol from L. digitata *frond*

Solvent (150 ml., except in expt. 4)	Extraction time, hr.*	Wt. of weed, g.	Mannitol extracted (% of total mannitol)	Ash extracted (% of total ash)	Mannitol recovered Found, % Ash	Mannitol	Yield as % of mannitol extracted
1. Methanol ..	2	5·008	77·9				
„ ..	3	5·008	80·4	37·5	0·35	97·9	48·7
2. Methanol ..	2	4·834	83·7				
„ ..	3	(all passes 64-mesh)	84·5	48·0	1·19	93·1	59·4
3. Methanol ..	2	5·031	75·6				
„ ..	3	(water 6·27%)	76·8	40·0	0·82	95·8	63·0
4. Methanol (132 ml., i.e. 55 ml./g. mannitol)	3	9·398 (all passes 64-mesh)	74·3	29·4	0·20	99·9	76·1
5. Methanol stirred in flask	2	10·009	63·2	23·8	0·64	98·1	51·4
6. Ethanol ..	2	5·013	68·9				
„ ..	3	5·013	68·9	17·8	5·93	92·3	66·6
7. Ethanol ..	2	5·053	65·2				
„ ..	3	(water 6·27%)	65·9	25·5	10·77	82·5	71·5
8. 90% Ethanol	2	5·035	88·6				
„	3	5·035	90·9	51·6	8·16	85·1	58·7
9. *n*-Propanol ..	2	5·001	66·3				
„ ..	3	5·001	66·3	7·1	1·90	94·4	72·4
10. *n*-Butanol ..	2	5·006	62·4				
„ ..	3	5·006	63·2	5·2	2·14	95·7	74·8
11. Pyridine	2	5·013	73·4				
„	3	5·013	74·2	—	—	—	—
12. Water	2	2·504	96·1	65·2	—	—	—

* With exception of experiment 5, all extractions were carried out in Haanen and Badum extractor.

Table III

Solvent extraction of mannitol from L. cloustoni *frond*

Solvent (55 ml./g. of mannitol in sample)	Extraction time, hr.*	Wt. of weed, g.	Mannitol extracted (% of total mannitol)	Ash extracted (% of total ash)	Mannitol recovered Found, % Ash	Mannitol	Yield as % of total mannitol extracted
1. Methanol, 78 ml.	3	9·156	72·8	36·3	0·8	95·5	73·1
2. Methanol, 116 ml.	3	15·13 (water, 9·52%)	82·8	38·2	0·6	92·0	73·2
3. Methanol, 424 ml.	3	50·08	63·8	28·4	0·2	96·5	59·8
4. Methanol, 424 ml.	3	55·45 (water, 9·52%)	64·2	30·8	0·2	95·5	51·1
5. *n*-Butanol, 430 ml.	3	50·63	28·6	0·0	0·6	99·8	85·3

* In experiments 1 and 2, the continuous Haanen and Badum extractor was used; in experiments 3 to 5, the weed was stirred in a flask, under reflux, with the boiling solvent.

Discussion of results

Experiments 1 and 2 (Table II) suggest that the amount of mannitol extracted from dried milled *L. digitata* frond in a standard extractor depends on the state of division of the weed, presumably because cell-wall hardening occurs when weed has been exposed to boiling anhydrous organic solvents, and the remainder of the mannitol is trapped within the cell and is not removable by further treatment with the solvent. Water, which does not produce this effect, removed practically all the mannitol in 2 hours but also removed 65% of the mineral matter. It is not possible, however, to separate the mannitol from such aqueous solutions by fractional crystallization, and separation can only be accomplished by evaporation to dryness and the use of methods given later in this paper.

The total amount of mannitol extracted by the alcohols from ground anhydrous *L. digitata* frond of definite particle size decreased with increase in boiling point of the alcohol; the solubility of the mineral matter also decreased similarly, while the amount of mannitol recovered by crystallization of the extract increased with increase in boiling point of the solvent (Expt. 1, 6, 9 and 10; Table II). By choosing the optimum methanol–mannitol ratio the yield of mannitol recovered from methanol was increased from 49% to 76% (Expt. 1 and 4, Table II). Furthermore, mannitol crystallizing from methanol appeared rarely to be contaminated with mineral matter, whereas that recovered from ethanol and *n*-propanol frequently contained ash. Similar results were obtained with *L. cloustoni* frond (Table III).

Pyridine removed approximately the same amount of mannitol as methanol but this solvent was not investigated further because of the difficulty of mannitol recovery, owing to the high solubility in pyridine.

Experiments 3, 7 and 8 (Table II) show that it is not essential for the weed to be anhydrous, although increase in water content of the solvent used results in increasing contamination of the recovered mannitol. Provided the correct ratio of alcohol to weed is used it is possible to extract the mannitol simply by stirring the weed in the presence of boiling methanol under reflux in a flask, although the percentage of mannitol extracted is less than when continuous extraction is used. This latter method has the great disadvantage, particularly with *L. digitata*, that the mannitol crystallizes out almost immediately from the boiling solvent, and cannot be easily separated from the weed residue.

In such experiments it is possible to recover 97% of the solvent by distillation of the mother liquor, leaving a residue of mannitol and salts. Attempts to isolate a further quantity of mannitol from this residue by a second extraction with boiling methanol in a flask have proved unsuccessful, the mannitol failing to crystallize from solution. The recovery of some of this mannitol might be effected, however, by conversion to a water-insoluble derivative as described in section 3(*b*).

2. Laminarin–mannitol–salt mixtures

Dilute-acid extraction of dried weed.—Laminarin, almost completely insoluble in organic solvents, can be extracted from dried milled brown seaweed with water. The addition of either hydrochloric or sulphuric acid to pH 2·4 greatly reduces the retention of water by the weed residue.

Work at present in hand in these laboratories is directed towards determining optimum conditions for the extraction of laminarin, but sufficient has been done to prove that the method is capable of removing most of the mannitol at the same time. A typical extraction is as follows.

Ground *L. digitata* frond [250·3 g.; Table I, (2)] was stirred with 0·09N-HCl (2500 ml.) and 40% formaldehyde (2·5 ml.) in the cold for 1 hour. The weed residue was centrifuged, washed with water (3 × 250 ml.), the centrifugate and washings were neutralized with caustic soda and evaporated at 45° C./15 mm. to dryness. This laminarin–mannitol–salt mixture was partially dried by heating at 55° C./12 mm. during 4 hours and completely dried *in vacuo* over phosphorus pentoxide, giving a hard brown solid (66·8% of the frond). The isolation of mannitol from mixtures of this type has been investigated and the results are given in Table IV.

Solvent extraction of mannitol from laminarin–mannitol–salt mixtures.—Work on the extraction of mannitol from dried milled weed having shown that methanol was the best solvent, this was the only solvent examined for the recovery of mannitol from these laminarin–mannitol–salt mixtures under conditions given in the following typical extraction.

Experimental and results

The anhydrous, finely powdered mixture from *L. digitata* frond (137·3 g., containing 32·41 g. mannitol) was refluxed with boiling methanol (1780 ml., i.e. 55 ml. per g. of mannitol) for 5½ hours

with stirring in a flask fitted with reflux condenser. The solution was filtered hot, and the insoluble residue washed with ether and dried to a dull-white powder.

The filtrate, in which crystals formed almost immediately, was kept overnight at 5° C., and the crystallized mannitol was filtered, washed with ether, and dried at 100° C. for 45 min. to a pure white powder. The filtrate was evaporated *in vacuo* to give the final extract, which was ground to a light-green hygroscopic powder.

The analysis of these fractions is given in Table IV.

Table IV

Extraction of laminarin–mannitol–salt mixture from L. digitata *with methanol*

Laminarin–mannitol–salt mixture						Methanol extract : crystallized mannitol (dry basis)				
			Found, %					Found, %		
Mixture, as % of frond	Ash	Mannitol	% of total mannitol	Laminarin	% of total laminarin	Mannitol, as % of mixture	Ash	Mannitol	Mannitol, as % of mannitol in mixture	Mannitol, as % of mannitol in frond
66·8	29·0	23·6	88·6	27·8	78·0	16·4	1·8	93·7	65·1	57·7

Insoluble residue						Final extract			
			Found, %					Found, %	
Residue, as % of mixture	Ash	Mannitol	Mannitol, as % of mannitol in mixture	Laminarin	Laminarin, as % of laminarin in mixture	Extract, as % of mixture	Ash	Mannitol	Mannitol, as % of mannitol in mixture
47·6	22·4	7·31	14·7	48·8	83·6	31·5	49·3	19·2	25·6

Discussion of results

The results recorded in Table IV show that mixtures of laminarin, mannitol and inorganic salts, provided they are completely anhydrous and finely powdered, can be extracted by refluxing with methanol in a flask. Boiling with 55 ml. methanol per g. of mannitol in the mixture for 5½ hours removed 85% of the mannitol present and 65% crystallized out from the methanolic filtrate, corresponding to 76% of the mannitol extracted from the mixture and 58% of the total mannitol in the original frond. The losses incurred in this isolation (calculated on the total mannitol present in the frond) are therefore : (1) 11% retained in the weed residue after the acid extraction, which could be reduced by more efficient washing and filtration ; (2) 13% retained in laminarin–mannitol–salt mixture which might be reduced by longer extraction ; and (3) 18% in the methanolic mother liquor. Attempts to recover the mannitol in the mother liquor, by removal of the solvent and extracting the residue again with the calculated amount of methanol, failed.

3. Mannitol–salt mixtures

Removal of laminarin from acid extracts.—Unpublished work in these laboratories has shown that with *L. cloustoni*, laminarin (80% of the total extracted) is precipitated when an acid extract is allowed to stand overnight, while with *L. digitata* the laminarin does not separate out from such extracts even after prolonged standing. On the other hand, the addition to these acid extracts of ethanol to 85% almost quantitatively precipitates the laminarin.

Preparation of mannitol–salt mixture.—As a preliminary to investigations 3(*a*), (*b*) and (*c*), and to provide starting material for these, *L. digitata* frond [Table I ; (1)] was treated under three sets of conditions as follows. The results obtained are recorded in Table V.

Experimental and results

(1) The weed (1010 g.) was stirred with 0·09N-hydrochloric acid (10 l.) and 40% formaldehyde (10 ml.) in the cold for 2 hours and left overnight to allow the weed residue to settle. The weed residue was filtered on cloth, washed twice with water (1 l. each time), the filtrate and washings were neutralized with caustic soda (28 ml. 40%), and evaporated at 40–50° C./20 mm. to a viscous solution (1500 ml.).

The solution was then treated with alcohol to 85% (w/w) (10·7 l.) and allowed to stand overnight. The precipitate A, containing the laminarin and fucoidin together with some of the salts and a small fraction of the mannitol, was filtered off, washed with alcohol and ether, and dried in a vacuum desiccator over phosphorus pentoxide to give a white powder.

(2) Higher yields of mannitol were obtained by centrifuging instead of filtering off the weed residue, e.g. *L. digitata* frond (413·2 g.) as in (1) above was stirred with 0·09N-hydrochloric acid

(4·5 l.) and 40% formaldehyde (4·5 ml.) in the cold for 4½ hours. The weed residue was centrifuged, washed with water (2 × 400 ml.), and the centrifugate was neutralized with sodium hydroxide and evaporated at 55° C./15 mm. to 540 ml. The viscous solution was then treated with alcohol to 85% (w/w) and allowed to stand overnight to allow the precipitate A to settle. The precipitate A was centrifuged off, washed with alcohol and ether, and dried.

(3) This experiment was carried out under identical conditions to (2) but with 0·09N-sulphuric acid substituted for hydrochloric acid.

In each experiment the alcoholic filtrate from precipitate A was evaporated at 35–45° C./20 mm. to give the mannitol–salt mixture B as a light-green solid. This was rendered anhydrous by drying *in vacuo* over phosphorus pentoxide. The analysis of solids A and B is given in Table V.

Table V

Preparation of mannitol–salt mixtures

	Expt. No.	% of dry frond	Ash	Found, % Mannitol	Laminarin	% recovery of total Mannitol	Laminarin
Alcoholic precipitate A	1	21·5	19·7	13·0	47·9	10·9	73·6
	2	22·3	24·3	10·7	—	9·4	—
	3	27·0	37·0	4·4	—	4·6	—
Residual mannitol–salt mixture B	1	36·8	42·0	43·1	—	62·3	—
	2	35·9	31·0	52·7	—	74·2	—
	3	35·7	29·7	54·9	—	76·9	—

3 (*a*). Solvent extraction of mannitol–salt mixtures

Methyl, ethyl, *n*-butyl and *n*-propyl alcohols, acetone, pyridine and concentrated hydrochloric acid were tested against mannitol–salt mixture B obtained as above.

Experimental and results

The following extraction procedure was adopted for Expt. 1–10 (Table VI):

The anhydrous powdered mixture B was continuously extracted with the boiling solvent for 5 hours in the Haanen and Badum extractor already described. The insoluble residue in the crucible was dried and weighed. The solution containing the extracted material was allowed to stand 24 hours at 5° C. and the precipitate, referred to in Table VI as crystalline mannitol, was filtered and weighed. The filtrate was then evaporated *in vacuo* to dryness to give the final extract. In Expt. 11 continuous extraction was replaced by simple extraction in a flask. The anhydrous powdered mixture B was refluxed with boiling methanol with stirring, and filtered hot to give the insoluble residue. The filtrate was treated as described for continuous extraction. The results obtained are recorded in Table VI.

Discussion of results

Of the solvents examined only methanol was found to be suitable. With *n*-butanol (Expt. 1), the mixture rapidly darkened, became syrupy and impermeable to the solvent.

With *n*-propanol (Expt. 2) the mixture darkened slightly but remained permeable to the solvent till the end. Despite this, mannitol extraction was incomplete and the product impure. Ethanol (Exp. 3) extracted more mannitol but dissolved more salts, and acetone was quite useless because of the low solubility of mannitol in this solvent (4 mg. per 100 ml. at 4° C.; 16 mg. per 100 ml. at boiling point). Pyridine was also found to be unsatisfactory as the mixture darkened and a dark-brown insoluble syrup was deposited.

Concentrated hydrochloric acid, although capable of separating pure mannitol–potassium chloride mixtures, gave no clear-cut separation with these mannitol–salt mixtures (Expt. 12). When mixture B was treated with cold acid for 25 hours, filtered to remove the insoluble residue, and the filtrate evaporated *in vacuo* to remove the acid, the resulting final extract was still contaminated with salts. Further processing would be necessary to isolate pure mannitol from this extract.

The optimum conditions of extraction with methanol are given in Expt. 11 (Table VI) where the mannitol–salt mixture was almost quantitatively dissolved by the methanol, and pure mannitol (ash 0·2%) crystallized out from the solution in 84·4% yield.

3 (*b*). Separation of mannitol from mannitol–salt mixtures as an insoluble derivative

The possibility of converting the mannitol in mannitol–salt mixtures into a water-insoluble derivative was investigated as a means of separating the mannitol and later reconverting it to the

Table VI

Solvent extraction of mannitol–salt mixtures

Expt. No.	Solvent and volume, ml.	Wt. of mixture taken, g.*	Extraction time, hr.	Insoluble residue: Weight as % of mixture	Found, % Ash	Found, % Mannitol	Mannitol, as % of total mannitol in mixture	Time of solution in refrigerator, days	Crystalline mannitol: Weight as % of mixture	Found, % Ash	Found, % Mannitol	Mannitol, as % of total mannitol in mixture	Final extract: Weight as % of mixture	Found, % Ash	Found, % Mannitol	Mannitol, as % of mannitol in mixture	Total mannitol accounted for
1	*n*-Butanol (150)	10·32	5	95·5	—	—	—	1	1·5	—	—	—	—	—	—	—	—
2	*n*-Propanol (150)	5·218	,,	49·3	64·2	24·0	27·5	,,	32·1	15·0	77·5	57·5	16·9	—	25·7	10·1	95·3
3	Ethanol (150)	5·623	,,	32·5	77·0	17·9	13·5	,,	36·8	12·4	66·3	56·6	29·7	—	29·7	20·5	90·6
4	Acetone (150)	5·530	,,	92·3	42·0	42·3	90·6	—	—	—	—	—	—	—	—	—	—
5	Pyridine (50)	5·139	,,	37·1	94·8	1·0	0·9	1	23·4	0·2	96·9	52·6	32·1	6·84	36·9	27·5	81·0
6	Methanol (150)	5·275	,,	1·5	—	—	—	,,	24·4	0·8	91·7	51·9	74·9	50·4	26·4	45·9	97·8
7	Methanol (150)	5·325	,,	1·2	—	—	—	3	32·1	1·4	92·8	69·0	66·7	55·0	18·0	27·8	96·8
8	Methanol (100)	5·517	4	1·3	—	—	—	,,	45·2	18·7	78·6	82·4	52·9	55·7	12·6	15·5	97·9
9	Methanol (125)	5·303	,,	1·7	—	—	—	,,	34·2	0·6	96·0	76·3	64·1	59·4	14·3	21·3	97·6
10	Methanol (146)	4·860	,,	2·6	—	—	—	,,	46·2	0·2	94·8	79·8	51·4	48·5	15·6	14·6	94·4
11	Methanol (162)	5·395	3	1·8	—	—	—	1	46·3	0·2	101·9	84·4	51·0	50·3	14·6	13·6	98·0
12	Conc. HCl (13·0)	12·08	25	32·3	99·7	0·1	0·1	—	—	—	—	—	70·4	9·8	57·6	94·0	94·1

* Mixture B (Expt. 1, Table V) used except in Expt. 10 and 11, where mixture B (Expt. 3, Table V) was used.

hexitol, and also as a means of preparing and isolating such derivatives, some of which are finding application in industry.

A literature survey showed that of the known insoluble derivatives, the triethylidene, tribenzylidene, triacetone, hexa-acetyl and hexanitro-derivatives showed the greatest promise. These have been prepared using mannitol–salt mixtures obtained both from the Laminariaceae and Fucaceae as starting materials. The methods given in the literature for pure mannitol have, in general, been adhered to, and the yields could possibly be improved by determining the optimum conditions applicable to mixtures of mannitol and salt.

Experimental and results

(i) *Mannitol–salt mixtures from the Laminariaceae*

Triethylidene mannitol.—The conditions worked out by Meunier (1903) were adhered to. The mannitol–salt mixture (mannitol, 52·7; ash, 31·0%; 146·2 g.) was stirred with concentrated hydrochloric acid (184 ml.) and paraldehyde (102 ml.) in the cold for 15 min., the crystalline mass diluted with water (2·5 l.), filtered, washed thoroughly with water (3 × 800 ml.), and dried to give a greenish-white powder (91·8 g.). Yield 83·4%; m.p. 170–171° C.; $[\alpha]_D$ − 72·3° [*c*, (g./100 ml.) 0·83 in $CHCl_3$]. Meunier (1903) obtained a 96·9% yield from pure mannitol. Bourne & Wiggins (1948) quote m.p. 172° and $[\alpha]_D$ − 72·3° (*c*, 0·8 in $CHCl_3$) for pure triethylidene mannitol.

Hydrolysis of triethylidene mannitol.—The conditions used by Wiggins (1946*a*) for the decomposition of 3 : 4-monoethylidene mannitol were employed.

Triethylidene mannitol (2·101 g.) was heated with N-H_2SO_4 (50 ml.) at 100° C. for 6 hours without a reflux condenser, the volume being maintained at 50 ml. by adding water at intervals. The solution was neutralized with barium carbonate, filtered and evaporated *in vacuo* to a crystalline mass. This was extracted with boiling *n*-butanol (400 ml.) for 45 min., filtered and placed in the refrigerator overnight to crystallize. Yield of mannitol, 93·2%; m.p. 160–162° C.; $[\alpha]_D$ − 0·2° (*c*, 4·73 in water). On a larger scale the mannitol could be recrystallized from water, and the acetaldehyde recovered by steam-distilling the hydrolysis mixture.

Tribenzylidene mannitol.—The method of Fischer & Fay (1895) was employed. The mannitol–salt mixture contained mannitol, 43·1; ash, 42·0%.

The mixture (65·25 g., containing 28·13 g. mannitol) was treated with concentrated HCl (56 ml.) and benzaldehyde (56 ml.) in the cold for 19 hours with occasional stirring. Water (800 ml.) was then added, the precipitate was filtered, washed thoroughly with water (3 × 250 ml.), alcohol (100 ml.) and ether (100 ml.), and dried to give a white powder (65·5 g.). Yield, 95·0%. M.p. 220-222° C.

A wide range of melting points has been reported for tribenzylidene mannitol: 207° C. by Meunier (1891), 218–222° C. by Fischer & Fay (1895) and 230–234° C. by Hatt & Hillis (1947).

Attempted hydrolysis of tribenzylidene mannitol.—This compound was found to be very stable to acid hydrolysis. When 2·005 g. was refluxed at 100° C. with N-H_2SO_4 (40 ml.) and ethanol (8 ml.) for 8 hours, very little of the solid dissolved. Neutralization, followed by evaporation and extraction with *n*-butanol as described for the triethylidene derivative, gave no crystalline mannitol.

Triacetone mannitol.—The method of Wiggins (1946*b*) was used. The mannitol–salt mixture contained mannitol, 43·1; ash, 42·0%.

The anhydrous, finely ground mixture (pass 64-mesh; 40·6 g.; containing 17·50 g. mannitol) was shaken for 16 hours with anhydrous acetone (220 ml.) and concentrated H_2SO_4 (1·8 ml.). The dark-brown solution was neutralized with anhydrous Na_2CO_3 and filtered; the residue was thoroughly washed with acetone, and the filtrate and washings evaporated *in vacuo* to a green crystalline solid (27·9) g. This product was purified by dissolving in ethanol (20 ml.) and pouring into water (500 ml.). The greenish-white crystals were filtered, washed with water, and dried (15·55 g.). Yield, 53·5%. M.p. 66–67° C.

Wiggins (1946*b*) obtained this derivative in 75% yield from pure mannitol, and reported a m.p. of 69–70° C.

A yield of 38·2% was obtained from the same mannitol–salt mixture by pouring the acetone solution after shaking for 16 hours directly into water (1 l.). This eliminates the neutralization with Na_2CO_3, evaporation to dryness and solution of the product in alcohol, but results in a lower yield.

Hydrolysis of triacetone mannitol.—Triacetone mannitol (2·467 g.) was heated under reflux with N-H_2SO_4 (50 ml.) at 100° C. for 2 hours, neutralized with barium carbonate and the filtrate was evaporated *in vacuo* to a mass of white crystals. This was extracted with boiling *n*-butanol (400 ml.) for 1 hour, filtered, and the solution placed in the refrigerator overnight to crystallize. Yield 92·9%. M.p. 162–164° C.

Hexa-acetyl mannitol.—The mannitol–salt mixture contained mannitol, 43·1; ash, 42·0%. The anhydrous finely-ground mixture (60·4 g.; containing 26·03 g. mannitol) was refluxed with acetic anhydride (416 ml.) and anhydrous sodium acetate (104 g.) for 40 minutes. The dark-brown mixture was poured into cold water (3 l.) with stirring, when the oily acetate solidified after a few minutes. The brown crystals were filtered, washed thoroughly with water (3 × 400 ml.), and dried (55·75 g.; yield 89·8%). This was purified by recrystallization from 50% (v/v) ethanol (800 ml.), to give 52·25 g. of mannitol hexa-acetate (yield 84·1%, based on weight of original mannitol). A second recrystallization from 50% ethanol (1 l.) gave 49·60 g. of crystals, which were still light brown in colour. Yield 79·9%. M.p. 122° C. [cf. m.p. of pure mannitol hexa-acetate 126° C. (Patterson & Todd, 1929)].

Hydrolysis of hexa-acetyl mannitol.—This was not attempted because of the problem of separating the salts introduced on deacetylation.

Mannitol hexanitrate.—This was prepared as described by Sokoloff (1879). The mannitol–salt mixture in this experiment contained ash, 34·8%; laminarin 7·97%; mannitol, 34·4%. To the mixture (1·123 g.), which contained 0·139 g. water, were added gradually ice-cold nitric acid (1·1 ml., sp. gr. 1·5) and ice-cold sulphuric acid (1·8 ml., sp. gr. 1·84), and after standing 5 minutes in ice, ice-cold water (50 ml.) was added and the semi-solid precipitate filtered off, washed with water and dried to give a light-brown solid A (0·747 g.). Yield 88·9%.

Nitrate A (0·747 g.) was recrystallized from boiling 70% ethanol (10 ml.) to give a white powder B. (0·385 g.; m.p. 91–94° C.). Nitrate B was recrystallized from 70% ethanol to give a powder C. Yield 38·5%; m.p. 102–106° C. Nitrate C was recrystallized from 70% ethanol to give a powder. Yield (based on weight of original mannitol), 35·9%; m.p. 103–107° C.

Patterson & Todd (1929) obtained a 75% yield from mannitol, and quote a m.p. of 112–113° C. for pure hexanitro mannitol. With pure mannitol, we obtained a yield of 75·0% after three recrystallizations from 70% ethanol, but the m.p. was still low (108–109° C.).

Hydrolysis of mannitol hexanitrate.—As with the hexa-acetate this was not attempted because of the difficulty of separating the soluble nitrates introduced on hydrolysis.

(ii) *Mannitol–salt mixtures from the Fucaceae*

Triethylidene mannitol.—The mannitol–salt mixture prepared from *Ascophyllum nodosum* [Table I, (4)] contained ash, 52·8% and mannitol, 23·0%. The mixture (1·640 g.) was treated with concentrated hydrochloric acid (0·9 ml.) and paraldehyde (0·5 ml.) in the cold for 15 min. Water (15 ml.) was then added, the solid filtered, washed three times with water, and dried *in vacuo* over P_2O_5 to a greenish-white powder (0·459 g.). Yield 85·2%; m.p. 167–169° C.; $[\alpha]_D$ − 65·0° (c, 0·846 in $CHCl_3$). This yield agrees well with that from the mannitol–salt mixture from *L. digitata* (yield, 83·4%).

The mannitol–salt mixture from *Pelvetia canaliculata* [Table I; (5)], containing ash, 51·7%; mannitol, 23·0%, was treated as above, giving a yield of 20·2% of impure triethylidene mannitol; m.p. 158–161° C., $[\alpha]_D$ − 59·7° (*c*, 0·804 in $CHCl_3$).

Tribenzylidene mannitol.—The mannitol–salt mixture (1·692 g.) from *Ascophyllum nodosum*, as above, was treated with water (0·15 ml.), concentrated HCl (0·8 ml.) and benzaldehyde (0·8 ml.) in the cold for 17 hours. Water (15 ml.) was then added, the solid was filtered, washed with water, alcohol (10 ml.) and ether (10 ml.), and dried to give a pure-white powder (0·548 g.). Yield 57·5%; m.p. 217–219° C.

An attempt to prepare the tribenzylidene derivative from the mannitol–salt mixture from *Pelvetia canaliculata* was unsuccessful. A solid was obtained, m.p. 235–238° C. Mixed m.p. with pure tribenzylidene mannitol (m.p. 215–216° C.), 205–208° C.

Discussion of results

The results obtained show that mannitol can be separated from a mixture of mannitol and salts by conversion to a water-insoluble derivative such as acetal, ketal or ester.

From *L. digitata* mannitol–salt mixtures the triethylidene, tribenzylidene, triacetone, hexanitro- and hexa-acetyl derivatives were obtained in 83, 95, 54, 36 and 80% yields respectively. From an

Ascophyllum nodosum mannitol–salt mixture the triethylidene derivative was obtained in 85% yield and the tribenzylidene mannitol in 58% yield, indicating that the bulk of the mannitol in this species is present as such and not in the anhydride form.

With *Pelvetia canaliculata* the triethylidene mannitol was obtained in only 20·2% yield, and it was found that the benzylidene derivative, which was obtained in very poor yield, was not tribenzylidene mannitol. These findings are in some agreement with the work of Haas & Hill (1929), who found it impossible to isolate crystalline mannitol from *Pelvetia canaliculata*, but were able to isolate the tribenzal derivative. They inferred that in this species mannitol is present to some extent as mannitan. They were able to isolate a second benzylidene derivative (m.p. 165–168° C.), which they believed, from its analysis, to be a dibenzal mannitan. It is interesting to note that the melting point of dibenzylidene styracitol (1 : 5-mannitan) has since been given as 163–165° C. (Asahina & Takimoto, 1931). From the point of view of obtaining mannitol from these compounds, the tribenzylidene, hexa-acetyl and hexanitro- derivatives are unsatisfactory—the tribenzal because of its great stability towards acid, and the esters because of the problem of separating salts introduced again on hydrolysis.

The preparation of the trimethylene derivative was not attempted because of the time required to get quantitative yields (Ness, Hann & Hudson, 1943) and because of its great stability. It could no doubt, however, be obtained from a mannitol–salt mixture, and its application in industry as a plasticizer may warrant such a preparation.

Triacetone mannitol is the most easily decomposed, and mannitol, after recrystallization from butanol, is obtained in 93% yield.

The triethylidene derivative is readily formed in good yield (83%) and fairly readily hydrolysed to give mannitol (93% yield), corresponding to 58% recovery from the original frond.

This method for the recovery of mannitol from seaweed has recently been patented (Scottish Seaweed Research Association, Black & Dewar, 1949).

3 (c). Separation of mannitol from mannitol–salt mixtures using ion-exchange resins

The cation-exchanger used in these experiments was Zeo-Karb 225, an efficient resin having a capacity of about 2 mg.-equiv. per ml. (back-washed and drained bed volume). The anion-exchangers employed were De-Acidite F and Amberlite IRA-400 (OH), strongly basic resins, the latter having a capacity of about 1 mg.-equiv. per ml. in column form.

The following procedure was adopted :

The mannitol–salt mixture (9·5 g.) was dissolved in water (200 ml.) to give a solution approximately 2% (w/v) with respect to salts. The filtered solution was passed through a column of Zeo-Karb 225 (70 ml. contained in an 0·64-in. diameter tube), and then through an equal volume of De-Acidite F contained in a similar tube. The columns were washed with water (400 ml.), the effluent evaporated *in vacuo* to dryness, and the crystalline solid obtained was analysed for mannitol and ash. With the Amberlite resin, only 7·1 g. of mixture were used, in order to bring the salt content well within the capacity of this resin. The results are given in Table VII.

Table VII

Demineralization of mannitol (43·1%)*–salt* (42·0%) *mixture*

Expt. No.	Wt. of mixture, g.	Volume of water, ml.	Cation-exchanger Anion-exchanger (70 ml. of each)	Time of passage through columns, min.	Analysis of demineralized solid: Wt. as % of mixture	Ash (450° C.), %	Ash (Bunsen) (' true ash '), %	Mannitol, %
1	9·515	200	Zeo-Karb 225 De-Acidite F	60	59·1	12·6	—	81·2
2	9·532	200	„	120	59·0	17·2	3·4	83·9
3	9·572	200	„	30	55·5	4·8	1·8	86·3
4	4·771	400	„	30	59·1	17·4	5·8	78·4
5	Mannitol (4·114 g.) NaCl (4·003 g.)	200	„	30	57·2	19·0	6·5 % NaCl in solid, 1·22	83·6
6	Mannitol (3·020 g.) NaCl (3·002 g.)	200	Zeo-Karb 225 Amberlite IRA-400 (OH)	30	48·9	5·2	2·1	95·5
7	7·115	200	„	30	49·6	7·9	2·8	92·1

The high yields of demineralized solid obtained with De-Acidite F (Expt. 1–5), compared with those with the Amberlite resin (Expt. 6 and 7), indicate that De-Acidite F is not stable to solutions of such high concentration and that some of the resin is being dissolved during acid removal. The low mannitol contents and the high ash figures at 450° C. with De-Acidite F confirm this view. This effect was minimized by reducing the time of contact to 30 minutes (Expt. 3), although decreasing the salt concentration to 0·5% (Expt. 4) had little effect on the stability of the resin. In Expt. 5 a solution of mannitol and sodium chloride was prepared, in which the concentrations of mannitol and mineral matter were similar to those in the algal mannitol–salt mixture. Again the final product had a high ash content at 450° C., while the percentage of sodium chloride (estimated with $AgNO_3$) was only 1·2.

Amberlite IRA-400 (OH) was found to be quite stable to 1·5% salt solutions. Expt. 6 and 7 show that a mixture containing equal parts by weight of mannitol and salts can be 97% demineralized by a single treatment with a cation-exchanger (Zeo-Karb 225) followed by an anion-exchanger (Amberlite IRA-400). The demineralized solid is then sufficiently high in mannitol to allow recrystallization from water. For example, the solid obtained in Expt. 7 was recrystallized from water to give an 85% yield of mannitol (allowing for the solubility of mannitol in water at the temperature of the crystallization). The long, needle-shaped crystals contained mannitol, 97·6; ash, 0·4%. One serious disadvantage to the use of ion-exchange resins for large-scale demineralizations of this sort is the high cost of Amberlite IRA-400 (OH). Not only is this an expensive resin initially, but once it is exhausted a large excess of regenerant is required to convert it to the hydroxyl form. About 10 volumes of 2N-sodium hydroxide are required for regeneration, so that in Expt. 7 about 55 g. NaOH was used for the isolation of 3 g. mannitol. Zeo-Karb 225 is a less expensive resin, has a higher capacity than the anion-exchanger, and requires only one volume of 2N-hydrochloric acid for regeneration (i.e. 16·2 g. concentrated hydrochloric acid, sp. gr. 1·16, for the isolation of 3 g. mannitol in Expt. 7.)

Although no experiments have yet been carried out, it may be possible to demineralize directly dilute aqueous acid extracts of weed to give a relatively pure laminarin–mannitol mixture, without the isolation or the mannitol–salt mixture. There would appear, however, to be certain difficulties: for example, dilute aqueous acid at pH 2·4, apart from extracting the bulk of the mannitol, laminarin and mineral matter from the frond, also removes some fucoidin and small quantities of soluble alginate. The alginate on passage through the cation-exchanger would be rendered insoluble and deposited on the resin, and would not be removed on regenerating the column with hydrochloric acid.

Summary

The following methods of isolating mannitol from brown marine algae have been investigated.

1. *Solvent extraction of dried ground weed.*—Extraction with water in a Haanen and Badum extractor is practically quantitative but the product is heavily contaminated with mineral matter and soluble polysaccharides.

With the anhydrous alcohols case-hardening of the milled weed occurs and for complete extraction fine grinding of the weed is essential. The percentage extraction of mannitol and salts from anhydrous *L. digitata* frond is given in that order in parentheses: methanol (80, 38); ethanol (69, 18); *n*-propyl alcohol (66, 7) and *n*-butyl alcohol (62, 5).

The use of aqueous ethanol or pyridine is not recommended. Aqueous ethanol, although it removes 91% of the mannitol, also removes 52% of the mineral matter, and the mannitol which crystallizes out is contaminated with salts. Although mannitol is fairly readily extracted by boiling pyridine, the recovery from this solvent is very low.

Methanol appears to be the best solvent, and by choosing the optimum methanol–mannitol ratio it is possible to extract 74% of the mannitol, and 76% of this crystallizes out as pure mannitol. This represents an overall yield of 57% from the original *L. digitata* frond. Ninety-seven per cent. of the methanol can be recovered on distillation.

2. *Mannitol separation from aqueous weed extracts.*—The recovery of mannitol from mannitol–laminarin–salt mixtures, obtained by evaporating to dryness aqueous acid extracts of brown algae, has been attempted using four different methods:

(*a*) Extraction with methanol, which removes 85% of the mannitol present, 76% of which crystallizes out in a pure form, corresponding to a 58% overall yield from the frond.

(*b*) Precipitation of the laminarin followed by solvent extraction of the residual mannitol–salt mixture. Methanol has given the best results, dissolving almost completely such mixtures, and pure mannitol crystallizes out from this solution in 84% yield, corresponding to a yield of 65% from the original weed.

(*c*) Separation from the mannitol–salt mixtures through the intermediate separation of water-insoluble derivatives, such as the triethylidene and triacetone compounds, followed by hydrolysis.

The triethylidene derivative, obtained in 83% yield and affording a 93% recovery of mannitol on hydrolysis, is the most promising, in that this represents an overall yield of 58% from the original weed.

In addition, derivatives such as tribenzal (95% yield), hexa-acetyl (80% yield) and hexanitro mannitol (36% yield) have been prepared, such compounds having an application in industry.

(*d*) Demineralization of mannitol–salt mixtures by means of ion-exchange resins. With Zeo-Karb 225 as cation-exchanger and Amberlite IRA–400 (OH) as anion-exchanger, it is possible to raise the mannitol content of a mannitol (43%)–salt (42%) mixture to 92% and reduce the ash to 2·8% in a single column treatment. The demineralized solid is then sufficiently pure to allow recrystallization from water to give pure crystalline mannitol. The high cost of regeneration of these resins is discussed.

Acknowledgments

The work described in this paper forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association, to whom the authors are indebted for permission to publish.

The assistance of Mr. W. J. Cornhill in the analytical work is also acknowledged.

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Received 27 November, 1950

References

Asahina, Y. & Takimoto, H. (1931). *Ber. dtsch. chem. Ges.* **64**, 1803.

Bashford, L. A., Thomas, R. S. & Woodward, F. N. (1950). *J. Soc. chem. Ind.* **69**, 337.

Berk, F. W. & Six, C. (1942). B.P. 545,672.

Black, W. A. P. (1948). *J. Soc. chem. Ind.* **67**, 165, 169, 172, 355; 1949, **68**, 183.

Bourne, E. J. & Wiggins, L. F. (1948). *J. chem.Soc.* 1933.

Fischer, E. & Fay, I. W. (1895). *Ber. dtsch. chem. Ges.* **28**, 1979.

Haas, P. & Hill, T. G. (1929). *Biochem. J.* **23**, 1005.

Hatt, H. H. & Hillis, W. E. (1947). *J. Coun. sci. industr. Res. Aust.* **20**, 207.

Meunier, J. (1891). *Ann. Chim.* (*Phys.*) **22**, (6), 412.

Meunier, J. (1903). *Bull. Soc. Chim. Fr.* **29**, (3), 735.

Ness, A. T., Hann, R. M. & Hudson, C. S. (1943). *J. Amer. chem. Soc.* **65**, 2215.

Patterson, T. S. & Todd, A. R. (1929). *J. chem. Soc.* 2876.

Scottish Seaweed Research Association, Black, W. A. P. & Dewar, E. T. (1949). B.P. Appl. 30,393.

Skroznikova, N. S. (1939). *Chem. Zbl.* **11**, 483; *Chem. Abs.* 1941, **35**, 3001

Sokoloff, N. (1879). *Ber. dtsch. chem. Ges.* **12**, 698.

Tanret, C. (1902). *Bull. Soc. Chim. Fr.* **27**, (3), 947.

Wiggins, L. F. (1946*a*). *J. chem. Soc.* 384.

Wiggins, L. F. (1946*b*). *J. chem. Soc.* 13.

Reprinted from the Journal of Applied Chemistry, 1951, 1, *pp.* 505–517

MANUFACTURE OF ALGAL CHEMICALS. III. LABORATORY-SCALE ISOLATION OF LAMINARIN FROM BROWN MARINE ALGAE

By W. A. P. BLACK, W. J. CORNHILL, E. T. DEWAR and F. N. WOODWARD

Methods for the isolation of laminarin from the common brown algae indigenous to Great Britain have been worked out on the laboratory scale with a view to the ultimate development of a process suitable for large-scale production. These methods comprise treating the fresh or dried milled weed with hydrochloric acid or sulphuric acid at pH 2·4 and isolating the laminarin from the acid extract.

Introduction

Laminarin, a glucose polymer composed entirely of β-D-glucopyranose units linked through the 1 : 3-positions, was first described by Schmiedeberg (1885), who isolated it from the Laminariaceae. It has since been studied by Krefting & Torup (1909), Kylin (1913), Gruzewska (1923), Colin & Ricard (1929), Lunde (1937), Nisizawa (1940), Le Gloahec & Herter (1940), and Barry (1938, 1942), and their work has been reviewed by Hassid (1944).

Barry showed that laminarin consisted of glucose units, and that after methylation and hydrolysis it gave 2 : 4 : 6-trimethyl glucopyranose. He concluded that laminarin consisted of a chain of 1 : 3-glucopyranose units bent into a spiral form, and an end-group assay using periodic acid indicated a chain length of 16 glucose units (Barry, 1942). A chain length of 20 glucose units has recently been given for laminarin from *Laminaria cloustoni* (Connell, Hirst & Percival, 1950).

Laminarin occurs only in the frond of the Laminariaceae and to a lesser extent in the Fucaceae, and is subject to marked seasonal variation (Black, 1948, 1950).

The only attempt yet made to isolate laminarin on a large scale is that of Le Gloahec & Herter (1940), who treated the seaweed with an alkaline-earth metal solution such as calcium chloride or barium chloride, separated the solution, and precipitated the laminarin by means of lead subacetate and sodium hydroxide.

The methods examined in the present investigation are all based on treatment of fresh or dried milled weed with dilute mineral acid, this being the first stage in the production of alginates (Bashford, Thomas & Woodward, 1950), and also one of the methods already worked out for the isolation of mannitol (Black, Dewar & Woodward, 1951). Treatment of the anhydrous frond with boiling organic solvents to extract mannitol (Black *et al.*, 1951) has also been shown to have no deleterious effect on the later isolation of laminarin by dilute-acid extraction.

With *Laminaria cloustoni*, and to a much lesser extent with *L. saccharina* and *L. digitata*, laminarin is deposited on standing from dilute-acid extracts of the frond, but with other species of brown algae examined it is much more soluble and can only be precipitated by the addition of alcohol or acetone. Laminarin appears, therefore, to exist in at least two forms, referred to in this investigation as the ' water-insoluble ' and ' water-soluble ' forms.

Species examined

The species used in this investigation, together with details of their composition, are recorded in Table I. After collection, the samples were dried on a rack at 25–30° C. for 48 hours and ground in a Christy and Norris mill, fitted with a 64-mesh screen, giving a product which practically all passed 90 mesh.

Table I

Composition of species examined

Species	Habitat	Date collected	% Chemical composition (dry basis) Ash	Mannitol	Laminarin	Alginic acid	Organic nitrogen
1. *Laminaria cloustoni* frond	Cullipool	Nov./Dec. 1945	15·0	13·3	28·6	13·1	2·30
2. *Laminaria cloustoni* frond	„	21.10.48	20·3	15·4	29·2	10·0	1·70
3. *Laminaria digitata* frond	Loch Melfort	22.10.45	19·3	17·8	23·8	19·3	0·68
4. *Laminaria saccharina* frond	„ „	5.9.45	18·2	19·6	26·5	15·6	0·66
5. *Ascophyllum nodosum*	Cullipool	8.6.45	17·3	9·0	4·3	24·4	1·33
6. *Fucus serratus*	Port Appin	24.8.45	21·4	14·8	9·4	18·0	1·42
7. *Fucus spiralis*	„ „	21.9.46	21·9	11·3	6·9	14·8	0·62
8. *Pelvetia canaliculata*	Atlantic Bridge	March 1949	24·1	8·5	2·5	—	1·55

Isolation of laminarin from dried milled *L. cloustoni* frond

Determination of optimum conditions of extraction

The material used in the experiments outlined below, except where otherwise stated, was dried milled *L. cloustoni* frond [Table I, (1)].

(1) *pH.*—Seven extractions were carried out in beakers at varying pH, with 50·18 g. frond in each case and an impeller stirrer at approximately 500 r.p.m. The results obtained are recorded in Table II. In expt. 1, the frond was heated with water (1 l.) at 70° C. for 4 hours; in expt. 2–7, the frond was stirred with the extracting liquid for 8 hours in the cold, and then allowed to stand for 16 hours. The mixture was then heated to 70° C. to dissolve the precipitated laminarin, and the weed residue was centrifuged, washed with the extracting liquid (2 × 100 ml.; 70° C.), air-dried at 65° C. for 1 day, weighed and analysed. The centrifugate and washings were stirred for 6 hours, allowed to stand 3 days, and the precipitated laminarin was centrifuged, washed with alcohol and ether, and dried *in vacuo* over phosphorus pentoxide. Yields of crude laminarin are expressed on a weight basis; several samples were analysed, and the results obtained varied between laminarin, 84 to 93%; ash, 1 to 2%. The chief disadvantage of extracting at high pH (expt. 1 and 2) was found to be the large volume of liquid retained by the weed residue, which renders centrifuging difficult and ordinary filtration almost impossible. Aqueous extraction removes soluble alginates which contaminate the laminarin precipitate; e.g., a typical precipitate was found to contain 4·1% alginic acid.

The yields of crude laminarin varied between 57 and 64%, and in all cases the laminarin left in the weed residue was less than 9%. Acid extraction, however, removed considerably more mineral matter than did aqueous extraction: for example, at pH 2·4 (expt. 4) 89·5% of the ash was extracted as compared with 64·4% at pH 4·9 (expt. 2), and 61·0% on aqueous extraction (expt. 1).

Sulphuric acid (expt. 6) was just as effective as hydrochloric acid in extracting laminarin, mannitol and mineral matter from dried milled frond, and a solution of calcium chloride (expt. 3) was no more efficient than hydrochloric acid at pH 2·4.

The results showed that within the experimental error the amount of laminarin extracted was independent of the pH, but filtering difficulties were encountered at the higher pH. Bearing in mind the possibility of the hydrolytic action of the dilute acid on the laminarin and its possible degrading effect on the alginic acid, pH 2·4 was chosen as optimum, being the highest pH which permitted easy filtration.

(2) *The ratio of acid to weed.*—The ratio of aqueous hydrochloric acid to weed was varied from 20, 10, 7·5, and 5·0 parts (by volume) of aqueous acid to 1 part (by weight) of weed, the concentration of acid being adjusted in each case to give pH 2·4. The other conditions of extraction were kept constant (expt. 4, Table II). The ratio of 10 parts of aqueous acid to 1 part of weed was found to be optimal; a lower ratio reduced the amount extracted (although this could have been compensated for by more thorough washing), and resulted in incomplete precipitation of the laminarin owing to the high viscosity of the solution. With the ratio of acid to weed greater than 10 to 1, the amount of laminarin deposited was also considerably reduced. 1 part of dried milled *L. cloustoni* frond [Table I, (1)], on mixing with 10 parts of 0·09N-HCl, gave pH of 2·4.

(3) *Extraction time and rate of stirring.*—In expt. 2–7 (Table II), the time of extraction was kept constant at 24 hours, but further work showed that this time could be considerably reduced; e.g., when the frond was stirred as before in the cold for 30 minutes with hydrochloric acid at

Table II

Extraction of L. cloustoni *frond at various pH values*

Expt. No.	Extracting liquid	pH of mixture	Wt. of weed residue as % of frond	Analysis of weed residue: Ash, %	Ash as % of total ash	Mannitol, %	Mannitol as % of total mannitol	Laminarin, %	Laminarin as % of total laminarin	Vol. of solution retained by weed residue, ml.	Wt. of crude laminarin precipitated, as % of total laminarin
1	Water (1000 ml.) at 70° C. for 4 hr. ..	—	39·8	14·7	39·0	2·2	6·6	5·0	6·9	200	57·2
2	0·01N-HCl (1000 ml.) in cold for 24 hr. ..	4·9	39·9	13·4	35·6	—	—	6·1	8·5	230	61·3
3	1% $CaCl_2$ (500 ml.) in cold for 24 hr. ..	5·4	41·5	17·4	48·1	3·0	9·4	5·6	8·1	120	59·1
4	0·05N-HCl (1000 ml.) in cold for 24 hr. ..	2·4	35·5	4·4	10·5	1·8	4·8	4·8	6·0	100	56·8
5	0·10N-HCl (1000 ml.) in cold for 24 hr. ..	—	34·2	2·7	6·1	1·7	4·4	6·4	7·6	70	57·4
6	0·10N-H_2SO_4 (1000 ml.) in cold for 24 hr.	1·7	34·9	3·1	7·1	2·0	5·2	3·2	3·9	80	63·9
7	0·27N-HCl (1000 ml.) in cold for 24 hr. ..	—	33·5	2·3	5·0	2·0	5·0	4·2	4·9	70	61·7

Table III

Effect of time of extraction and rate of stirring

Rate of stirring, r.p.m.	Time of extraction, min.	Wt. of crude laminarin, as % of total laminarin
200	5	39·6
500	30	43·0
500	20	43·6
500	10	45·0
500	5	43·0
1000	2	40·9
2000	1	39·3

Table IV

Effect of degree of fineness and temperature of extraction

Expt. No.	Weed size	Temperature of extraction, ° C.	Time of extraction, min.	% of total laminarin extracted	Precipitated laminarin: Wt. as % of total laminarin	Found, % Ash	Found, % Laminarin
1	Passes 16 mesh Retained 30 „	15	60	60·0	42·8	1·1	90·6
2	Passes 16 „ Retained 30 „	70	30	84·8	58·6	1·3	86·5
3	Passes 30 „ Retained 60 „	15	60	73·9	55·9	1·1	91·9
4	Passes 30 „ Retained 60 „	15	120	81·0	61·3	1·5	86·0
5	Passes 60 „	15	60	87·4	71·2	2·2	85·0

pH 2·4, 77% of the mineral matter, 85% of the mannitol and 91% of the laminarin was extracted; the yield of crude laminarin was 57·6%. (Found: laminarin, 87·5; ash, 1·0%.)

A further advantage of reducing the extraction time to 2 hours or less is that the laminarin does not precipitate in the presence of the weed, and it is then unnecessary to heat the mixture to 70° C. before centrifuging or filtering off the weed residue.

For further experiments on the rate of stirring and the minimum time of extraction, the stirrer used was of the 'fluid emulsifier' type with a shaft at an angle of 10° to the vertical, and the propeller blades set ¼ in. from the bottom of a 1-l. flask. In each experiment the dried milled frond (35·0 g.) was added to 0·09N-HCl (350 ml.) contained in the flask, and stirred at varying speeds in the cold for the appropriate time. After centrifuging, the weed residue was washed once with 0·09N-HCl (35 ml.), and the centrifugate and washings were allowed to stand for 3 days, and the precipitated laminarin isolated as before. The results obtained are shown in Table III; the lower yields obtained here as compared with Table II are due to less efficient washing.

The minimum times in Table III for a given speed were those required to 'wet' the weed thoroughly at that speed. The results indicate that, provided the frond is finely ground (all passes 60 mesh), the extraction of laminarin is a solution effect, and instantaneous as soon as the weed is thoroughly 'wetted.' The minimum extraction time, therefore, is the time required to 'wet' the weed, and this will depend on the moisture content of the dried weed, and possibly the presence or absence of a wetting agent.

With increase in particle size of the milled frond, the time required to wet the weed appears to be less, owing to the absence of 'caking,' but the rate of extraction of laminarin is also considerably less with increased particle size (see below).

(4) *Degree of fineness of dried milled frond and temperature of extraction.*—The material used was dried *L. cloustoni* frond [Table I, (2)], which was milled through a 16-mesh screen. Three samples of different particle size were prepared from this material, and analysed separately:

Weed size	Ash, %	Laminarin, %
Passes 16 mesh .. .. Retained 30 „	17·8	27·5
Passes 30 mesh .. .. Retained 60 „	19·0	30·9
Passes 60 mesh	27·4	24·3

The milled frond (25 g.) was stirred at 500 r.p.m. with 0·09N-HCl (250 ml.) for the appropriate time. The weed residue was centrifuged, washed with water (2 × 50 ml.), and air-dried at 65° C. for 1 day. The centrifugate and washings were stirred for 6 hours, allowed to stand for 3 days, and the precipitated laminarin was isolated as before. The weed residues and laminarin precipitates were analysed, and the results are recorded in Table IV.

The results indicate that with cold acid extraction fine milling (all passes 60 mesh) is essential if the bulk of the laminarin is to be removed in one hour or less (expt. 5). The extraction, however, depends also on the temperature, and with coarse ground weed (expt. 2) 30 minutes at 70° C. removes 85% of the total laminarin.

Heating, even with dilute acid, is not recommended, because of the possible degrading action on the alginate in the weed residue. One hour at 70° C., however, with hydrochloric acid at pH 2·4 has been found to have no hydrolytic effect on the laminarin present, but the effect on the alginate has not been investigated.

This method of extracting the water-insoluble form of laminarin has recently been patented (Scottish Seaweed Research Association, Black & Dewar, 1948).

Factors affecting the precipitation of laminarin

In an attempt to speed up the separation of the 'water-insoluble' laminarin from acid extracts the effect of the following factors has been investigated:

(1) *Temperature.*—The yield of 'insoluble' laminarin was not increased by allowing the acid solution to stand in the refrigerator at 5° C. instead of at room temperature.

(2) *pH.*—An acid extract of *L. cloustoni* frond, as previously described, was divided into portions and their pH adjusted with sodium hydroxide or hydrochloric acid. The results are recorded in Table V.

pH appears to have very little effect on the rate of precipitation, although slightly more is precipitated at pH 10, and this is contrary to the observations of Gruzewska (1923) and Barry (1938). Gruzewska (1923) found the precipitation of laminarin to be accelerated by the presence of acids and retarded by alkalis; Barry (1938) states that in presence of hydrochloric acid laminarin is deposited in 1½ hours, whereas the same solution to which a small amount of dilute sodium hydroxide had been added remained clear for 12 days.

Table V

Effect of pH on the precipitation of laminarin

pH	Wt. of laminarin precipitated as % of total laminarin			Total wt. of laminarin precipitated as % of total laminarin
	After 1 hr. stirring	After standing overnight	After 3 days	
2·6	9·9	15·8	12·6	38·3
4·1	8·9	18·1	8·2	35·2
6·9	11·8	22·4	Nil	34·2
10·2	11·6	18·1	12·5	42·2

(3) *Effect of seeding and stirring.*—An acid extract of *L. cloustoni* frond (35 g.; 350 c.c. 0·09N-HCl) was divided into 3 portions to which exactly 1 g. of laminarin was added, and the mixture stirred for different times. Stirring at speeds from 500 to 2000 r.p.m. for 30 minutes or less had no effect on the precipitation of the laminarin, 96% of the amount added as seed only being recovered. At 500 r.p.m. after 1 hour 6·7% of the total laminarin was deposited, and after 1¾ hours 8·5%. After standing overnight the amount deposited at the various speeds was identical. The rate of stirring appears, therefore, to have no noticeable effect on the rate of deposition of laminarin from acid solutions.

(4) *Effect of addition of possible precipitating agents.*—The following substances were added in varying amounts: acetone, sodium alginate, mannitol, borax, sodium chloride, sodium percarbonate, mercuric chloride, sodium hexametaphosphate, and triethanolamine, but in no case was the speed of precipitation increased nor the amount of final precipitate better than normal. In many cases the addition of such substances retarded precipitation; e.g., the solubility of laminarin appeared to be increased by the addition of sodium chloride and mannitol, while the addition of acetone up to 40% (v/v) almost completely retarded precipitation.

Seeding, stirring for an hour and allowing to stand overnight appeared to be the most satisfactory.

Complete precipitation of laminarin with alcohol

Because of the low yields of 'insoluble' laminarin (57–64% of crude product), precipitated from the aqueous acid solution on standing, an attempt was made to isolate the remainder, i.e. the 'soluble laminarin' by adding alcohol.

L. cloustoni frond (25·97 g.) was stirred with 0·09N-HCl (250 ml.) in the cold for 2 hours. The mixture, in which laminarin was beginning to deposit, was centrifuged, the weed residue washed with 0·05N-HCl (2 × 50 ml.) and air-dried at 65° C. for 1 day. The centrifugate was stirred for 3 hours, allowed to stand for 3 days, and the insoluble laminarin A isolated. The aqueous acid solution was then treated with alcohol to bring the concentration of alcohol to 85% (w/w), and the precipitated 'soluble' laminarin B isolated and dried. The final alcoholic solution was neutralized with NaOH and evaporated at 60° C./15 mm. to dryness (final extract). The various fractions were analysed and the results are recorded in Table VI.

Table VI

Complete precipitation of laminarin with alcohol

Fraction	Wt. as % of weed	Found, % Ash	Found, % Laminarin	Laminarin as % of total laminarin
Weed residue	45·3	7·4	13·4	21·2
Laminarin A	15·3	1·2	92·5	49·4
,, B	6·6	12·1	67·3	15·5
Final extract	37·1	46·1	2·9	3·8

The analysis shows that the soluble laminarin fraction (laminarin B) is impure, the high ash content (12·1%) suggesting that it is contaminated with fucoidin and/or salts. Analysis of the final extract shows it to be almost free from laminarin, indicating that the laminarin is completely precipitated by 85% alcohol.

Isolation of soluble laminarin

Dried milled frond [Table I, (2); 1001 g.] was stirred with 0·09N-HCl (10 l.) and 40% formaldehyde (15 ml.) in the cold for 30 minutes. The weed residue A was filtered on cloth, washed with water (3 × 1 l.) and dried.

The filtrate and washings were stirred for 3 hours, allowed to stand for 3 days, and the insoluble laminarin B centrifuged, washed with alcohol and ether, and dried to a white powder. The centrifugate was neutralized with caustic soda, and evaporated at 45° C./20 mm. to 1500 ml. The brown solution was then treated with alcohol to 85% (w/w) concentration, and the impure soluble laminarin C centrifuged, washed with alcohol and ether and dried to a brown solid. The

alcoholic centrifugate was evaporated *in vacuo* at 45° C. to give the mannitol–salt mixture D as a light-brown, sticky solid, which was dried *in vacuo* over P_2O_5.

The analysis of these fractions is recorded in Table VII.

Table VII

Analysis of fractions from L. cloustoni *frond*

Fraction	Wt. as % of frond	Ash, %	Mannitol, %	Mannitol as % of total mannitol	Laminarin, %	Laminarin as % of total laminarin
Weed residue A	40·4	14·6	3·3	8·7	11·0	15·2
Laminarin B	18·7	1·1	2·1	2·5	83·8	53·7
Laminarin C	12·6	21·0	6·5	5·3	53·6	23·1
Mannitol–salt mixture D ..	30·9	37·9	38·7	77·8	3·4	3·6

Laminarin C (113·6 g.) was stirred with hot water (1250 ml.) and an insoluble bulky residue was centrifuged and washed with water (200 ml.). The dark-brown centrifugate and washings were then passed through a Zeo-Karb 225 column (280 ml.), and the column washed with water (400 ml.). The effluent and washings (1770 ml.) were treated with alcohol to 85% (w/w) concentration, when laminarin C1 was precipitated as a semi-colloid. This was coagulated by adding NaCl (2·0 g. in 10 ml. water), and the precipitate was centrifuged, washed with alcohol and ether, and dried to a pink powder (64·2 g., i.e. 56·5% of laminarin C).

$[\alpha]_D$, 11·7° [*c* (g./100 ml.), 1·113 in water]. Found : laminarin, 76·6 ; ash, 1·3%

Effect of previous treatment with boiling alcohols on the extraction of laminarin

One of the methods developed for the extraction of mannitol from dried milled weed (Black, Dewar & Woodward, 1951) employs boiling organic solvents such as methanol or *n*-butanol, and the effect of this treatment on the later extraction of laminarin has been studied.

The milled frond [Table I, (2)] was first dried at 105° C. for 16 hours and then refluxed with the boiling solvent (55 ml. per gram of mannitol in frond) for 3 hours. The weed residue was filtered off, washed with alcohol and ether, dried at 100° C. for 45 minutes, and extracted with 0·09N-HCl (10 ml. per gram of original frond) in the cold or at 70° C. for 30 minutes. The acid extract, after centrifuging from the weed residue, was stirred for 3 hours, allowed to stand for 3 days, and the precipitated laminarin isolated in the usual way. The results are recorded in Table VIII.

Table VIII

Extraction of laminarin after treatment with boiling organic solvents

Expt. No.	Treatment of frond	Temperature of HCl extraction, ° C.	Precipitated laminarin: Wt. as % of total laminarin	Found, % Ash	Found, % Laminarin
1	Nil	15	72	—	—
2	Methanol	15	65	1·3	85·5
3	,,	70	64	—	—
4	*n*-Butanol	15	70	1·5	87·5
5	,,	70	79	1·8	84·3

The results show, within the limits of recovery of laminarin, that previous treatment of the anhydrous frond with boiling alcohols has no deleterious effect on the subsequent extraction of laminarin with dilute acid.

The water content of laminarin

In many cases, particularly with the water-soluble form from *L. digitata* reported later, the laminarin content of samples by the standard method of analysis has been between 82 and 88% with ash contents as low as 1% and less. Different strengths of acid and varying times of hydrolysis were tried but offered no explanation. Water estimations were carried out by Percival & Ross (private communication) on a sample which had a laminarin content of 89·4%, calculated on a moisture figure of 8·8% after 48 hours' drying at 40° C./0·005 mm.

Active-hydrogen estimations by the micro-Zerewitinow method were carried out on the original sample and on the dried specimen, giving active hydrogen on 'wet' sample of 1·76% equivalent to 15·8% water, and 0·68% on 'dry' sample equivalent to 6·1% water. Correcting the figure of 89·4% for a moisture content of 6·1%, the purity becomes 95·2%. The equilibrium moisture content of laminarin appears, therefore, to be of the same order as that for starches.

Isolation of laminarin from fresh *L. cloustoni* frond

Preliminary experiments with fresh frond (laminarin, 15·1% ; dry basis), cut with scissors into pieces ½ in. × ¼ in., indicated that stirring the weed (250 g.) with 0·09N-HCl (500 ml.) in

the cold for 2 hours removed only 21% of the laminarin, whereas after 25 hours in the cold 35% was dissolved out.

Shredding with a ham slicer into $\frac{1}{32}$-in. slices, however, gave better results. A 4-hour, cold, dilute-acid treatment of the fresh frond (laminarin, 23·6%; dry basis) as above removed 65% of the total laminarin, whereas 2 hours' heating at 70° C. and then standing overnight extracted 83%.

Further experiments were carried out with frond which had been minced in a Reliance meat mincer fitted with a $\frac{1}{16}$-in. plate. In each experiment, 250 g. of the minced frond, equivalent to 59·5 g. dry matter (found: ash, 28·0; mannitol, 9·2; laminarin, 24·5% dry basis), were stirred with 0·125N-HCl (500 ml.) and 40% formaldehyde (1 ml.), giving a resulting normality of hydrochloric acid in the solution of 0·09. After treatment as described in Table IX, the weed residue was filtered on cloth, washed with water (2 × 100 ml.), dried, weighed, milled through a 64-mesh screen and analysed. The filtrate and washings were stirred for 3 hours, allowed to stand 2 days, and the precipitated laminarin was centrifuged, washed with alcohol and ether and dried *in vacuo* over phosphorus pentoxide. In expt. 5 (Table IX) a sample of the fresh frond was dried and milled through a 64-mesh screen, and then treated with 0·09N-HCl (690 ml. for 60 g. dried milled frond) as above; in this way the extraction of laminarin from fresh frond was compared directly with that from dried milled frond. The results are recorded in Table IX.

Table IX

Processing of fresh L. cloustoni *frond*

Expt. No.	Treatment of weed	pH of solution	Analysis of weed residue: Wt. as % of weed	Laminarin, %	Laminarin as % of total laminarin	Wt. of crude laminarin as % of total laminarin
1	In cold for 30 min.	5·4	63·5	21·1	54·7	30·9
2	Heated at 70° C. for 60 min. ..	5·1	50·4	8·4	17·3	59·6
3	Heated at 70° C. for 120 min. ..	5·1	47·3	7·0	13·5	58·4
4	Minced frond allowed to stand in cold for 23 hr., then treated with acid in cold for 1 hr.	5·3	63·6	27·6	71·6	13·7
5	Dried milled frond heated at 70° C. for 60 min.	5·2	43·8	3·1	5·5	58·1

The crude laminarin precipitated in expt. 2 was analysed and found to contain laminarin, 88·1; ash, 1·4%.

From these results, and from a further set of experiments not reported here, the following conclusions can be drawn. It is desirable to have the fresh weed shredded as finely as possible, and to process it immediately before case-hardening occurs as in expt. 4 (Table IX). Cold acid extraction for 30 minutes (expt. 1) removes approximately 50% of the laminarin, while heating at 70° C. for 30–60 minutes extracts 79–83% from finely minced frond (expt. 2). Sixty per cent. of the total laminarin (expressed on weight of crude product) is precipitated from the aqueous acid filtrate on standing.

After drying and milling, dilute-acid extraction at 70° C. for 60 minutes (expt. 5) removed 95% of the laminarin, but the yield of precipitated laminarin was the same as that from the fresh weed extractions (expt. 2 and 3).

Although the normality of the hydrochloric acid in the solution was 0·09N., as in all previous work with dried milled weed, the final pH of the mixture was 5, indicating that this sample had considerable buffering action. However, with fresh *L. cloustoni* frond lifted from the same habitat one month later, the pH of the mixture under the same conditions was 2·1; the buffering effect, therefore, would appear to vary with the time of year. These variations in pH seem to have little effect on the extraction of mannitol and laminarin from either fresh or dried milled weed, but they have some influence on the removal of mineral matter; low pHs give a more efficient extraction of the ash.

Isolation of laminarin from dried milled *L. saccharina* frond

Laminarin from *L. cloustoni* has been shown to consist of two fractions: (1) the relatively pure insoluble form which is precipitated on standing from dilute acid solution, and (2) the impure soluble form, which is precipitated from the mother liquor with alcohol. Laminarin from *L. digitata*, as will be shown later, has been found to give only the soluble form in normal conditions. Laminarin from *L. saccharina* forms an insoluble fraction which, however, only constitutes about 17% of the total laminarin, as compared with over 50% from *L. cloustoni* under the same conditions.

The conditions worked out for the extraction of laminarin from *L. cloustoni* were found to be satisfactory for *L. saccharina*, i.e. 1 part (by weight) of weed to 10 parts (by volume) of 0·09N-HCl gave a resulting pH of 2·5 with no filtering difficulties.

A typical extraction of *L. saccharina* was carried out as follows :

The weed [Table I, (4); 26·02 g.] was stirred with 0·09N-HCl (250 ml.) in the cold for 2 hours. The solution was centrifuged, the weed residue washed with 0·05N-HCl (2 × 50 ml.), and air-dried at 65° C. for 1 day. The dark brown centrifugate was seeded with laminarin (from *L. cloustoni*), stirred for 5 hours and allowed to stand for 3 days, and the insoluble laminarin A isolated and dried. Alcohol was then added to the mother liquor to 85% (w/w) concentration, and the precipitated laminarin B isolated. The fractions were analysed, and the results are given in Table X.

Table X

Analysis of laminarin fractions from L. saccharina

Fraction	Wt. as % of weed	Found, % Ash	Mannitol	Laminarin	% of total laminarin
Weed residue	35·9	8·1	2·7	7·2	9·8
Laminarin A	5·9	1·5	—	78·2	17·4
„ B	23·3	6·4	—	77·2	67·8
					95·0

The extraction removed from the frond 84% of the mineral matter, 95% of the mannitol, and 90% of the laminarin : 17% of the total laminarin separates from the acid solution after 3 days' standing, and 68% is precipitated with alcohol.

Counter-current extraction with HCl at pH 2·5

For the isolation of a quantity of laminarin from this species, a counter-current extraction was carried out as follows : 101 g. dried milled frond [Table I, (4)] were stirred with 0·09N-HCl (1 l.) in the cold for 2 hours, and the weed residue centrifuged and washed with water (2 × 100 ml.). A second 101-g. batch of weed was then brought into contact with the centrifugate, the pH adjusted to 2·5 with 10N-HCl, and stirred in the cold for 2 hours. After separation at the centrifuge and washing of this weed residue, a third batch of weed was extracted with the centrifugate in the same way. After four 101-g. lots had been extracted, the final centrifugate was treated with alcohol to 85% concentration, when the insoluble and soluble fractions were precipitated together and isolated. The analysis of the crude laminarin is shown in Table XI.

Table XI

Analysis of crude laminarin from counter-current extraction of L. saccharina *frond*

Fraction	Dry wt., g.	Wt. as % of frond	Found, % Ash	Laminarin	Laminarin as % of total laminarin
Laminarin	124·8	30·9	8·4	74·7	87·1

Isolation of laminarin from dried milled *L. digitata* frond

The conditions of extraction worked out for *L. cloustoni*, and found applicable to *L. saccharina*, were also suitable for the extraction of *L. digitata*. When an acid extract of milled frond of this species [Table I, (3)], prepared as described for *L. saccharina* (see above), was seeded with laminarin (*L. cloustoni*), stirred for 13 hours and allowed to stand for 2 days, no laminarin was deposited. The extract was then made up to 85% concentration with alcohol, and the precipitated laminarin isolated as before. The alcoholic centrifugate was neutralized with NaOH and evaporated at 60° C./15 mm. to dryness (final extract). The fractions were analysed and the results are recorded in Table XII.

Table XII

Analysis of fractions from L. digitata *frond*

Fraction	Wt. as % of weed	Found, % Ash	Mannitol	Laminarin	Laminarin as % of total laminarin
Weed residue	41·2	10·5	3·5	6·7	11·6
Laminarin	23·0	8·2	—	72·8	70·2
Final extract	39·7	34·8	—	8·0	13·3
					95·1

Weak acid extraction at pH 2·5 in the cold for 2 hours has removed 78% of the mineral matter, 92% of the mannitol, and 88% of the laminarin from the weed, and 70% of the total laminarin in the frond was precipitated with alcohol (85% w/w). The laminarin like the soluble laminarin from *L. cloustoni* is impure, the ash content indicating the presence of fucoidin. An appreciable amount (13%) of the laminarin is not precipitated by 85% alcohol, indicating the presence of an even more soluble form than is present in *L. cloustoni* or *L. saccharina*.

Counter-current extraction with HCl at pH 2·5

For the isolation of a quantity of this crude ' soluble ' laminarin, a counter-current extraction, with four 100-g. batches of frond [Table I, (3)], was carried out exactly as described for *L. saccharina*. The analysis of the crude laminarin is shown in Table XIII.

Table XIII

Analysis of crude laminarin from counter-current extraction of L. digitata *frond*

Fraction	Dry wt., g.	Wt. as % of frond	Found, % Ash	Found, % Laminarin	Laminarin as % of total laminarin
Laminarin	87·2	21·9	10·7	69·3	63·7

Purification of the crude laminarin from L. digitata

(1) *Reprecipitation with alcohol.*—The work described above has shown that the laminarin from *L. digitata* is not precipitated from aqueous acid solutions on standing for 2 days. The possibility did exist, however, that the mineral matter, mannitol, etc., present in the solution were hindering the precipitation. A 2·9% (w/v) aqueous solution of crude laminarin (relatively free from soluble salts and mannitol) was acidified, stirred for 5 hours and allowed to stand several days, but no precipitate formed.

A more concentrated solution, containing 7·0 g. crude laminarin (ash, 10·1 ; laminarin, 64·9%) in 50 ml. water and 2 drops 10N-HCl, was left overnight, but again nothing precipitated. This solution was diluted to 120 ml., alcohol added to 85% concentration, and the reprecipitated laminarin was isolated (5·5 g.). Found : ash, 8·9 ; laminarin 72·1%. Reprecipitation gives very little purification, as would be expected if fucoidin is the impurity.

(2) *Fractional precipitation with alcohol.*—Crude laminarin (2·9 g. ; ash, 10·1 ; laminarin, 64·9%) was dissolved in water (100 ml.) and alcohol added slowly with stirring. Precipitation commenced after adding 200 ml., i.e. 61% (w/w) concentration. At 70% the precipitate was centrifuged and isolated (fraction A, Table XIV). Alcohol was then added to the centrifugate to 85%, and fraction B isolated.

Table XIV

Fractional precipitation with alcohol

Fraction	Dry wt., g.	Found, % Ash	Found, % Laminarin	Laminarin as % of total in crude laminarin
A	2·36	9·0	67·2	84
B	0·32	3·5	80·1	14

Further attempts to purify by fractional precipitation were unsuccessful.

(3) *Precipitation with lead acetate and barium hydroxide.*—In their preparation of fucoidin, Percival & Ross (1950) extracted *F. vesiculosus* with hot water, and after precipitating the soluble alginates with lead acetate added barium hydroxide, and isolated the fucoidin as an insoluble barium hydroxide–lead hydroxide complex. The possibility of purifying laminarin, by removing small quantities of fucoidin in this way, has been investigated.

Crude laminarin (2·9 g. ; ash, 10·1 ; laminarin, 64·9%) was dissolved in hot water (50 ml.) and lead acetate solution [3·0 g. $Pb(OAc)_2,3H_2O$ in 10 ml. water] added, followed by cold saturated barium hydroxide (45 ml.) until the solution was just pink to phenolphthalein. The bulky white precipitate A was centrifuged, washed with cold water (2 × 30 ml.), and the centrifugate treated with excess H_2SO_4 (4N. ; 7 ml.) to remove Ba^{2+}. The $BaSO_4$ was centrifuged and washed with boiling water (2 × 15 ml.), and the centrifugate was treated with alcohol to 85% concentration. Only a small precipitate formed (0·093 g. ; ash, 63·6%), which was chiefly inorganic.

The white precipitate A was suspended in water (100 ml.), H_2SO_4 (4N. ; 10 ml.) added and the solution stirred for 3 hours to decompose the complex. The lead and barium sulphates were centrifuged, washed with boiling water (2 × 20 ml.), and alcohol added to the centrifugate to 85% concentration. The precipitate B was isolated as a white powder : 1·89 g. ; $[\alpha]_D$, 11·4° [*c* (g./100 ml.), 1·259 in water]. Found : ash, 1·0 ; laminarin, 83·5% ; i.e., 84% of the laminarin in the initial crude product.

A larger-scale experiment with 27·7 g. crude laminarin (ash, 10·7 ; laminarin 69·3%) yielded 17·3 g. purified product C (ash, 3·0 ; laminarin, 82·0%). Attempted purification of C by dissolving in water and reprecipitating with alcohol was unsuccessful.

In the purification experiments the laminarin tended to form a colloid in 85% alcohol, particularly when pure, but this was coagulated immediately on the addition of a small quantity of sodium chloride solution.

These experiments show that laminarin, as well as fucoidin, is precipitated with lead acetate

and barium hydroxide, but the treatment, nevertheless, has effected considerable purification of the crude product.

(4) *Treatment with a cation-exchange resin.*—It has been found that the free acid derived from fucoidin, obtained by treating an aqueous solution of fucoidin with the cation-exchanger Zeo-Karb 225, is not precipitated from aqueous solution on adding alcohol to 85% (w/w) concentration. The possibility of purifying laminarin in this way has been investigated; the crude product is dissolved in water, passed through a cation-exchanger column, and then alcohol is added, when the laminarin is precipitated and the free fucoidin acid remains in solution. This treatment also converts inorganic sulphates, which are precipitated at 85% alcohol concentration, to free sulphuric acid. This has proved to be the most successful method of purifying soluble laminarin yet examined. The following procedure was adopted. Crude laminarin (154·5 g.; laminarin, 72·5; ash, 7·3%) was dissolved in water (1800 ml.), and the solution was passed through a Zeo-Karb 225 column (140 ml. in a 0·64-in. diameter tube) for 6 hours, and the column was washed with water (150 ml.). When the strongly acidic effluent from the column was treated with alcohol to 85% concentration, laminarin separated as a semi-colloid, which was coagulated by the addition of sodium chloride (2·0 g. in 10 ml. water). The white precipitate was centrifuged off and isolated (134·1 g.). Found: ash, 1·3; laminarin, 82·2%.

Insoluble laminarin from L. digitata *frond*

It has been shown above that an acid extract of this species gives no insoluble laminarin fraction after 13 hours' stirring and 2 days' standing. A small quantity of insoluble laminarin, however, can be isolated after a prolonged period of stirring, as described below.

The frond [Table I, (3); 25·60 g.] was stirred with 0·09N-HCl (250 ml.) and 40% formaldehyde (0·25 ml.) at 70° C. for 30 minutes. The weed residue was centrifuged, washed with water (2 × 25 ml.), and the centrifugate and washings were seeded with insoluble laminarin (from *L. cloustoni*) and stirred for 8-hour periods during 14 days. A white precipitate slowly separated from solution. This was centrifuged, washed with alcohol and ether, and dried to a white powder, the analysis of which is shown in Table XV.

The results show that this insoluble fraction is not as pure as that from *L. cloustoni*.

Isolation of laminarin from dried milled Fucaceae

Extraction of F. serratus, A. nodosum *and* F. spiralis *with dilute acid*

The compositions of the materials investigated are given in Table I (5, 6 and 7).

Experiments to determine the optimum pH for the extraction of *A. nodosum* were carried out as for dried milled *L. cloustoni* frond. It was found that the highest pH to give reasonable centrifuging conditions was 1·9, and this was obtained by mixing 1 part (by weight) of weed with 10 parts (by volume) of 0·16N-HCl. Extraction of *A. nodosum* at pH 1·9 in the cold for 24 hours, exactly as described for *L. cloustoni* frond, removed 86% of the mineral matter, 84% of the mannitol and 70% of the laminarin.

For a comparison of the three species, 25 g. of each weed were stirred in the cold with 0·16N-HCl (250 ml.) for 2 hours, the weed residue centrifuged, washed with 0·05N-HCl (2 × 50 ml.) and air-dried at 65° C. for 1 day. The centrifugate and washings were then treated with alcohol to 85% concentration, and the precipitated material centrifuged, washed with alcohol and ether, and dried. The alcoholic solution was neutralized with sodium hydroxide and evaporated *in vacuo* to give the final extract. The results for the three species are recorded in Table XVI.

The results clearly indicate that the precipitates isolated at 85% alcohol concentration contain very little laminarin, the high (from *F. serratus*) being 24·7%. The high ash contents of these precipitates suggest they contain a fair proportion of fucoidin, and this has been confirmed, as described later, for *A. nodosum*.

Isolation of laminarin from Fucus serratus

Dried milled weed [Table 1, (6); 251 g.] was stirred with 0·16N-HCl (2·5 l.) and 40% formaldehyde (2·5 ml.) in the cold for 6 hours, and allowed to stand overnight. The weed residue was filtered on cloth, washed with water (2 × 250 ml.), and the filtrate and washings neutralized with caustic soda and evaporated *in vacuo* to 750 ml. The brown solution was treated with alcohol to 50% (v/v) concentration and the precipitate A was centrifuged, washed with alcohol and ether, and dried. The centrifugate was then treated with alcohol to 80% (w/w) concentration, and the precipitate B isolated. The analyses of A and B are given in Table XVII.

Precipitate B (9·175 g.) was dissolved in hot water (100 ml.), centrifuged to remove an insoluble residue, the clear centrifugate passed through a Zeo-Karb 225 column (70 ml. in 0·64-in. diameter tube), and the column washed with water (130 ml.). The strongly acidic, yellow effluent was treated with alcohol to 85% concentration, when precipitate B.1 was deposited. This was

Table XV

Analysis of insoluble laminarin from L. digitata *frond*

Fraction	Dry wt., g.	Wt. as % of frond	Found, % Ash	Found, % Laminarin	Laminarin as % of total laminarin
Insoluble laminarin	1·498	5·27	3·3	77·1	17·1

Table XVI

Hydrochloric acid extraction of Fucaceae in the cold for 2 hours

Species	pH of mixture	Analysis of weed residue: Wt. as % of weed	Ash, %	Ash as % of total ash	Laminarin, %	Laminarin as % of total laminarin	Precipitate at 85% alcohol: Wt. as % of weed	Ash, %	Laminarin, %	Laminarin as % of total laminarin	Analysis of final extract: Wt. as % of weed	Ash, %	Laminarin, %	Laminarin as % of total laminarin	Total laminarin accounted for, %
Fucus serratus	2·1	60·3	6·9	19·5	4·7	30·2	6·9	26·3	24·7	18·0	41·0	52·2	5·2	22·7	70·9
Ascophyllum nodosum	1·9	67·1	8·1	31·6	2·8	44·3	5·5	26·5	9·6	12·4	32·3	55·3	4·8	36·1	92·8
Fucus spiralis	1·4	55·0	8·0	20·1	5·8	46·6	10·4	28·6	17·6	26·5	51·3	64·5	3·0	22·3	95·4

Table XVII

Analyses of precipitates A and B from F. serratus

Fraction	Wt. as % of weed	Found, % Ash	Found, % Laminarin	Found, % Fucose
Precipitate A	6·3	37·5	6·4	7·1
Precipitate B	4·1	30·7	—	8·5

Table XVIII

Analysis of laminarin from F. serratus

Wt. as % of total laminarin	$[\alpha]_D$ [*c* (g./100 ml.),] 1·20 in water	Found, % Laminarin	Found, % Ash
19·3	−13·3°	93·5	0·2

Table XIX

Analysis of alcohol precipitate from A. nodosum

Wt. as % of weed	Ash	Mannitol	Found, % Laminarin	Total SO_4	Fucose	Laminarin as % of total laminarin
18·8	33·9	2·8	6·56	21·2	22·0	28·9

centrifuged, washed with alcohol and ether, and dried to a white powder (4·06 g.), which proved to be pure laminarin from its analysis in Table XVIII.

Counter-current extraction of Ascophyllum nodosum *with HCl at pH* 1·9

For the isolation of a quantity of material precipitated with alcohol, a counter-current extraction, using five 50·17-g. lots of weed and 500 ml. 0·16N-HCl, was carried out as described for *L. saccharina*. The weed was extracted each time in the cold for 24 hours and stirred during the first few hours. At the end of the extraction time, the mixture was heated to 70° C. and the weed residue was centrifuged and washed with 0·16N-HCl (2 × 100 ml.; 70° C.). The final centrifugate was neutralized with sodium hydroxide evaporated *in vacuo* to 400 ml., and alcohol was added to 60% (w/w) concentration. The precipitate was centrifuged, washed with alcohol and ether, and dried to a brown powder (47·2 g.). The analysis is shown in Table XIX.

The results show that this precipitate is very much richer in fucoidin than it is in laminarin.

Attempted isolation of laminarin from Pelvetia canaliculata

By the method used for *F. serratus* a small sample of material has been isolated from *Pelvetia canaliculata* [Table I, (8)] (yield 11% of the total laminarin; ash, 0·3%; laminarin, 77·9%; $[\alpha]_D$, + 21·9° in water) which does not agree with any of the modifications of laminarin so far isolated. This will be further investigated and reported later.

Summary

A method has been worked out and patented for the extraction of laminarin from fresh and dried milled brown marine algae. It consists essentially in treating the weed with hydrochloric or sulphuric acid at pH 2·4 and isolating the laminarin from the acid extract.

In agreement with the work of previous investigators, laminarin has been found to exist in at least two modifications: (1) a form which separates out from aqueous solutions; and (2) a form soluble in water but precipitated by alcohol at 85% concentration. The possibility of other forms soluble in 85% alcohol is not excluded.

The water-insoluble form is present to the extent of 55–65% of the total laminarin in *L. cloustoni* frond, 17% in *L. saccharina* frond, and is absent under normal conditions in *L. digitata* frond and the Fucaceae.

Optimum conditions have been worked out for the extraction of laminarin from *L. cloustoni*, and consist of stirring for 30 minutes in the cold 1 part (by weight) of the dried milled frond (all passing 64 mesh) with 10 parts (by volume) of acid (hydrochloric or sulphuric) at pH 2·4; or with the fresh, finely minced weed, stirring for 60 minutes at 70° C. This treatment removes about 90% of the laminarin, mannitol and mineral matter.

From *L. cloustoni* frond 55–65% of the total laminarin separates out from the acid extractant in an almost pure form (laminarin, 84–93; ash, 1%), while the impure water-soluble form is precipitated by alcohol. Purification is effected with a cation-exchange resin.

With *L. saccharina* frond, dilute-acid extraction removes 84% of the mineral matter, 95% of the mannitol, and 90% of the laminarin; 17% of the total laminarin separates from the acid solution after 3 days' standing and 68% is precipitated with alcohol (85% concentration).

With *L. digitata* 85% alcohol precipitates 70% of the total laminarin in the water-soluble form from an acid extract of the frond.

The water-insoluble form can be further purified by 'recrystallization' from hot water, but the soluble form is more difficult to purify. The most successful method is to treat an aqueous solution with a cation-exchange resin, whereby fucoidin and inorganic salts, present as impurities, are converted to free acids. Pure soluble laminarin is then precipitated from the acid solution with alcohol.

The extraction of soluble laminarin from the Fucaceae has also been investigated, and a pure sample has been isolated from *F. serratus* in 18% yield.

The specific rotations of the soluble laminarins from *L. cloustoni*, *L. digitata* and *F. serratus* are identical (− 11° to − 13° in water). Laminarin from *Pelvetia canaliculata*, however, has a positive rotation (+ 22° in water).

The percentage of laminarin soluble in 85% alcohol from *L. cloustoni* is 4%, from *L. saccharina* 5%, from *L. digitata* 13%, and from the Fucaceae over 20%, although these figures may be governed to some extent by the presence of mineral matter and other carbohydrates in the extractant.

Laminarin dried at 40° C. under high vacuum (0·005 mm.) still contained 6·1% water, indicating that the equilibrium moisture content is of the same order as that for starches.

Acknowledgments

The work described in this report forms part of the programme of research and development

on seaweed undertaken by the Scottish Seaweed Research Association, to whom the authors are indebted for permission to publish.

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Received 19 January, 1952

References

Barry V. C. (1938). *Sci. Proc. R. Dublin Soc.* **21**, 615 ; 939, **22**, 59 ; 1941, **22**, 423.
Barry, V. C. (1942). *J. chem. Soc.* p. 578.
Bashford, L. A., Thomas, R. S. & Woodward, F. N. (1950). *J. Soc. chem. Ind.* **69**, 337.
Black, W. A. P. (1948). *J. Soc. chem. Ind.* **67**, 165, 355 ; 1949, **68**, 183.
Black, W. A. P. (1950). *J. Mar. biol. Ass. U.K.* **29**, 54.
Black, W. A. P., Dewar, E. T. & Woodward, F. N. (1951). *J. appl. Chem.* **1**, 414.
Colin, P. & Ricard, R. (1929). *C. R. Acad. Sci., Paris* **188**, 1449.
Connell, J. J., Hirst, E. L. & Percival, E. G. V. (1950). *J. chem. Soc.* p. 3494.
Gruzewska, M. (1923). *Bull. Soc. Chim. biol., Paris* 5, 216.
Hassid, W. Z. (1944). *Ann. rev. Biochem.* **13**, 80.
Krefting, A. & Torup, R. (1909). *Pharmacia* **6**, 151.
Kylin, H. (1913). *Z. physiol. Chem.* **83**, 171 ; 1915, **94**, 337 ; 1918, **101**, 236.
Le Gloahec, V. C. E. & Herter, J. R. (1940). U.S.P. 2,188,092.
Lunde, G. (1937). *Z. angew. Chem.* **50**, 731.
Nisizawa, K. (1940). *Sci. Rep. Tokyo Bunrika Daigaku* [B] **5**, 9.
Percival, E. G. V. & Ross, A. G. (1950). *J. chem. Soc.* p. 717.
Schmiedeberg (1885). Gesellschaft deutscher Naturforscher und Arzte, Leipzig, Tageblatt der Versammlund, No. 58, p. 427.
Scottish Seaweed Research Association, Black, W. A. P. & Dewar, E. T. (1948). B.P. Appl. 30,553.

Reprinted from the Journal of the Science of Food and Agriculture, 1952, No. **3**, *pp.* **122–129**

MANUFACTURE OF ALGAL CHEMICALS. IV.*—Laboratory-Scale Isolation of Fucoidin from Brown Marine Algae

By W. A. P. BLACK, E. T. DEWAR and F. N. WOODWARD

Methods for the extraction and isolation of fucoidin from the common brown algae indigenous to Great Britain have been worked out on the laboratory scale, with a view to the ultimate development of a process suitable for large-scale production. Extraction involves treatment of the weed with hydrochloric acid at 70° for one hour at pH 2·0–2·5. The crude fucoidin is isolated by fractional precipitation with alcohol, and purified by treatment with formaldehyde.

Introduction

Fucoidin, a polyfucose ethereal sulphate occurring in the Phaeophyceae, was first described

* Part III : *J. appl. Chem.* 1951, **1**, 505 ; Part II : *J. appl. Chem.* 1951, **1**, 414 ; Part I : *J. Soc. chem. Ind.* 1950, **69**, 337.

and named by Kylin (1913), who prepared (Kylin, 1915) it from *Laminaria digitata* and showed that it contained the methylpentose, L-fucose, by isolating the phenylhydrazone on hydrolysis. He claimed that pentoses were also present in the hydrolysate. Bird & Haas (1931) obtained from *L. digitata* fronds a product containing ash, 30·9%, and sulphate on hydrolysis, 30·3%, and considered fucoidin to be a carbohydrate ethereal sulphate since the total sulphate on hydrolysis was approximately double that found in the ash. Lunde, Heen & Öy (1937) prepared fucoidin by precipitating the droplets exuded from fresh *L. digitata* fronds in alcohol. Their product contained ash, 26–30 ; sulphate in ash, 17–19 ; total sulphate, 35·5–37·7 ; and methylpentose (by distillation with hydrochloric acid), 33–37%. The ash consisted chiefly of sodium sulphate, with small quantities of potassium, calcium and magnesium sulphates. Since only about 80% of the molecule could be accounted for by this analysis, Lunde *et al.* (1937) proposed the formula $(R{\cdot}R^1{\cdot}O{\cdot}SO_2{\cdot}OM)_n$ for fucoidin, where R is fucose (as $C_6H_{10}O_4$), R^1 is unknown, and M may be Na, K, $Ca_{0·5}$ or $Mg_{0·5}$.

Another water-soluble polysaccharide closely related to, if not identical with, fucoidin was isolated from *Macrocystis pyrifera* by Hoagland & Lieb (1915) and shown to contain L-fucose and a high proportion of calcium and sulphate. Nelson & Cretcher (1931) showed the presence of an ethereal sulphate grouping and confirmed that fucose was the only sugar obtained after hydrolysis. Their product, however, had a uronic acid content of 2·6%, which was considered to be due to contamination with alginate.

Percival & Ross (1950) prepared fucoidin from *Fucus vesiculosus, F. spiralis, Himanthalia lorea* and *L. cloustoni*, by extraction with boiling water for 24 hours, removal of alginates and proteins with lead acetate, and precipitation of fucoidin as a lead hydroxide complex by addition of barium hydroxide. The resulting complex was decomposed with dilute sulphuric acid, and the fucoidin was isolated after prolonged dialysis and several treatments with Filter Cel. The various specimens gave similar analytical data, but the purest (from *H. lorea*) contained : fucose on hydrolysis (method of Cameron, Ross & Percival, 1948), 43·9, (chromatographic method of Flood, Hirst & Jones, 1948), 48·4 ; total sulphate, 32·4 ; metals, 6·9 ; ash, 22·6% ; $[\alpha]_D$—140° in water. The ash was mainly calcium sulphate. When allowances were made for adsorbed water and ethanol, which were found to be still retained by the polysaccharide after drying at 40°/0·1 mm. for 18 hours, the following figures were obtained : fucose on hydrolysis (chromatographic method), 56·7 ; total sulphate, 38·3 ; metals, 8·2%. Small quantities of uronic acid (3·3%), galactose (4·1%) and xylose (1·5%) were also detected. These workers believe that the principal constituent of fucoidin is a polyfucose monosulphate and that the other constituents arise from adventitious impurities. A calcium polyfucose monosulphate, $(C_6H_9O_3{\cdot}SO_4{\cdot}Ca_{0·5})_n$, would give on hydrolysis : fucose (as $C_6H_{12}O_5$), 66·9 ; total sulphate, 39·2 ; calcium 8·2%.

Conchie & Percival (1950) methylated fucoidin from *F. vesiculosus*, and on hydrolysis the carbohydrate portion was found to contain L-fucose, 3-methyl fucose and 2 : 3-dimethyl fucose, roughly in the proportions 1 : 3 : 1. The main residue in fucoidin was therefore believed to be a 1 : 2-α-fucopyranose unit carrying a sulphate group on C_4. They advanced two possible theories of accounting for the free fucose and 2 : 3-dimethyl fucose residues in methylated fucoidin : (1) Some fucose residues might carry two sulphate groups, whereas others (linked 1 : 4) are unsubstituted by sulphate groups :

$$\begin{array}{l} \qquad\qquad\qquad\quad SO_4^- \\ \qquad\qquad\qquad\quad 3 \\ —1F2—1F2—1F4—1F2—1F2— \\ \quad 4 \quad 4 \qquad\quad 4 \quad 4 \\ SO_4^- \; SO_4^- \qquad SO_4^- \; SO_4^- \end{array} \quad (F = \text{L-fucopyranose})$$

(2) Another possible explanation is that the free fucose originates from branching points at C_3 carrying terminal groups having free hydroxyls on C_2 and C_3 :

$$\begin{array}{l} \qquad\quad F_4SO_4^- \\ \qquad\quad 1 \\ \qquad\quad 3 \\ —1F2—1F2—1F2—1F2— \\ \quad 4 \quad 4 \quad 4 \quad 4 \\ SO_4^- \; SO_4^- \; SO_4^- \; SO_4^- \end{array}$$

These workers state, however, that a great deal of work must yet be carried out before the constitution of fucoidin is settled.

Fucoidin was considered by Kylin (1915) to be a cell-wall mucilage of the brown algae, particularly rich in the laminarias. It is now known, however, that fucoidin is present in all

common brown seaweeds, but to a much greater extent in the Fucaceae than the Laminariaceae, and undergoes a seasonal variation similar to alginic acid and cellulose, the other constituents of the cell-wall (Black, paper in preparation).

Apart from any industrial use which may be found for fucoidin itself when available in quantity, the polysaccharide should prove a much more suitable source of L-fucose than the present rather tedious process from the whole weed (Hockett, Phelps & Hudson, 1939). Although the Phaeophyceae are the best source of this sugar, L-fucose is also present in gum tragacanth (James & Smith, 1945), sea-urchin eggs (Vasseur, 1948), blood-group substances (Bray, Henry & Stacey, 1946) and frog-spawn mucin (Folkes, Grant & Jones, 1950).

The samples of fucoidin prepared in this investigation were dried either in a vacuum over phosphorus pentoxide or in an oven at 100–105° overnight, and therefore must still contain the bound water and ethanol revealed by Percival & Ross (1950). No correction has been made for these adsorbed solvents.

Species examined

The species used in this investigation, together with details of their composition, are recorded in Table I. After collection, the samples were dried on a rack at 25–30° for 48 hours and ground in a Christy and Norris mill, fitted with a 64-mesh screen. Fucose (as $C_6H_{12}O_5$) was estimated throughout by the improved colorimetric method (Black, Cornhill, Dewar, Percival & Ross, 1950), and the other constituents by the methods previously employed by one of the authors (Black, 1948). Because of the uncertainty of the exact fucose content of fucoidin, it is not yet possible to convert combined fucose figures into fucoidin. Ashing was carried out at 450° for 4–6 hours in a Wild Barfield furnace.

Table I

Composition of species examined

Species	Habitat	Date collected	% Chemical composition (dry basis)				
			Fucose (as $C_6H_{12}O_5$)	Ash	Mannitol	Laminarin	Alginic acid	Organic nitrogen
1. *Pelvetia canaliculata*	Atlantic Bridge	Mar. 1949	11·2	24·1	8·5	2·48		1·55
2. *Ascophyllum nodosum*	Cullipool	June 1945	9·0	17·3	9·0	4·27	24·4	1·33
3. *F. vesiculosus*	Port Appin	Nov. 1946	9·7	20·5	13·7	4·85	15·2	1·18
4. *F. vesiculosus*	Port Appin	Dec. 1945	10·1	26·0	10·6	2·6	15·0	1·20
5. *L. cloustoni frond*	Cullipool	Oct. 1948	3·14	20·3	15·4	29·2	10·0	1·70

Determination of optimum conditions of extraction

Several extractions were carried out to determine the effect of pH, temperature, extraction time and the ratio of extracting liquid to weed. The results obtained are recorded in Table II. An impeller type of stirrer was used in Expt. 1–7, and the rate was kept constant at approximately 500 r.p.m.

The dried milled weed was treated for the required time with the extracting liquid as shown in Table II at the pH and temperature indicated. After extraction, the weed residue was centrifuged, washed with water, air-dried at 65°, weighed and analysed. In Expt. 7, the weed was stirred with 0·17N-hydrochloric acid (200 ml.) at 70° for 1 hour, centrifuged and washed. The weed residue was then treated with water (150 ml.) to give the same volume as in the first extraction, the pH was reduced to 2·3 with 10N-hydrochloric acid (1·0 ml.), and the mixture was stirred at 70° for 1 hour. After centrifuging and washing, the weed residue was again treated with water (150 ml.), 10N-hydrochloric acid (0·5 ml.) added to pH 1·9, stirred at 70° for 1 hour, and the weed residue centrifuged and washed. Expt. 12 was carried out in the same way, except that water only was used in each extraction.

Extraction in the cold, even at the low pH of 1·5 (Expt. 2), removes very little fucoidin, and treatment with alkali at 75° is little better (Expt. 1). Stirring with hydrochloric acid at 70° for 1 hour at pH near to 2·5 extracts about 50% of the total fucoidin (Expt. 3, 4 and 5), whereas three acid extractions remove more than 80% (Expt. 7). The importance of controlling pH is shown in Expt. 6, where conditions identical to those in Expt. 5 existed except for pH, and the fucoidin extracted was only 22% as compared with 51% in Expt. 5.

As found in the preparation of laminarin (Black, Cornhill, Dewar & Woodward, 1951), the

Table II

Effect of pH, temperature, extraction time and ratio of extraction liquid to weed on the extraction of fucoidin from P. canaliculata *and* F. vesiculosus

Expt. No.	Species	Wt. of weed, g.	Extracting liquid and conditions	pH of mixture	Vol. of water washings, ml.	Wt. of weed residue as % of weed	Analysis of weed residue				Fucose extracted as % of total fucose
							Ash, %	Ash as % of total ash	Fucose, %	Fucose as % of total fucose	
1	*F. vesiculosus* Table I (3)	20·71	Stirred with 0·1N-NaOH (200 ml.) at 75° for 1 hr.	9·1	2 × 80	55·5	20·9	56·5	12·3	70·4	29·6
2	*P. canaliculata* Table I (1)	248·0	Stirred with 0·16N-HCl (2500 ml.) at 15° for 6 hr.	1·5	2 × 500	64·6	10·9	34·7	13·9	80·2	19·8
3	*P. canaliculata* Table I (1)	24·92	Stirred with 0·1N-HCl (250 ml.) at 70° for 1 hr.	2·7	2 × 50	59·4	12·6	31·1	9·5	50·4	49·6
4	*F. vesiculosus* Table I (3)	24·18	Stirred with 0·1N-HCl (250 ml.) at 75° for 1 hr.	2·5	2 × 50	61·2	8·1	24·3	6·9	43·5	56·5
5	*F. vesiculosus* Table I (4)	250·1	Stirred with 0·17N-HCl (2500 ml.) at 70° for 1 hr.	2·3	2 × 750	57·2	10·1	22·2	8·6	48·7	51·3
6	*F. vesiculosus* Table I (4)	252·0	Stirred with 0·1N-HCl (2500 ml.) at 70° for 1 hr.	4·5	2 × 750	61·4	16·2	38·2	12·8	77·8	22·2
7	*F. vesiculosus* Table I (4)	20·46	Stirred 3 times with HCl (200 ml.) at 70° for 1 hr.	1·9–2·5	1 × 80 after each extraction	44·0	3·5	5·9	4·23	18·4	81·6
8	*P. canaliculata* Table I (1)	25·23	Heated with water (250 ml.) at 100° for 3 hr.	5·7	2 × 60	55·9	18·9	43·8	9·0	44·9	55·1
9	*F. vesiculosus* Table I (3)	20·11	Heated with water (200 ml.) at 100° for 7½ hr.	5·8	2 × 80	48·0	13·9	32·5	8·0	39·6	60·4
10	*F. vesiculosus* Table I (3)	20·32	Heated with water (400 ml.) at 100° for 7½ hr.		2 × 80	42·8	14·5	30·3	7·3	32·2	67·8
11	*F. vesiculosus*, Table I (3)	10·21	Heated with water (200 ml.) at 100° for 15 hr.		2 × 40	41·0	13·2	26·4	5·3	22·4	77·6
12	*F. vesiculosus*, Table I (4)	20·39	Heated 3 times with water (400 ml.) at 100° for 7½ hr.		2 × 80 after each extraction	46·3	20·0	35·6	6·82	31·3	68·7

Table III

Fraction	Wt. as % of weed	Ash, %	Fucose, %	Fucose as % of total fucose	Fucose as % of fucose extracted
Precipitate B	6·60	22·2	11·1	7·6	12·5
Fucoidin C	13·6	26·4	31·3	43·9	72·7

chief disadvantage of aqueous extraction is the large volume of liquid retained by the weed residue, which renders the separation of the residue from the solution difficult. With water, however, there is less chance of degrading the fucoidin by partial hydrolysis than when acid at 70° is used. Aqueous extraction at 100° for 3 to 7½ hours removes 55 to 60% of the fucoidin (Expt. 8 and 9), although more efficient extractions can be obtained by increasing the water : weed ratio (Expt. 10), the extraction time (Expt. 11), or the number of extractions (Expt. 12).

From these experiments, hydrochloric acid extraction at 70° for 1 hour at or near pH 2·5 appeared to be the most satisfactory, three such treatments being necessary to remove 80% of the fucoidin.

Isolation of crude fucoidin from *F. vesiculosus*

The centrifugate and washings from Expt. 9 (Table II) were evaporated at 50°/20 mm. to dryness, and the dark-brown glass (11·15 g.) redissolved in water (100 ml.). The solution was treated with alcohol (25 ml.) to 20% (v/v) concentration, and the precipitate B was centrifuged, washed with alcohol (ethanol) and ether, and dried to a brown powder. This precipitate contains most of the soluble alginate. The alcoholic centrifugate was then treated with alcohol (125 ml.) to 60% (v/v) concentration, and the crude fucoidin C was centrifuged and isolated as above as a brown powder. The small quantity of laminarin present is not precipitated at this concentration of alcohol. The analysis of these fractions is given in Table III.

Effect of adding formaldehyde to seaweed extracts

When the combined centrifugates and washings from Expt. 12 (Table II), to which 40% formaldehyde (1·0 ml.) had been added as preservative, were evaporated *in vacuo* at 50° to dryness, the resulting glass was found to be only partially soluble in boiling water (100 ml.). The dark-brown residue A was centrifuged, washed with hot water (2 × 35 ml.), alcohol and ether, and dried. The centrifugate and water washings (130 ml.), which were now almost colourless, were treated with alcohol (56 ml.) to 30% (v/v) concentration, and precipitate B was isolated as a white powder. The alcoholic centrifugate was then treated with alcohol (139 ml.) to 60% (v/v) concentration, and fucoidin C isolated as a white powder. The analysis of these fractions is recorded in Table IV.

Table IV

Fraction	Wt. as % of weed	Ash, %	Fucose, %	Fucose as % of total fucose	Fucose as % of fucose extracted	Total sulphate	$[\alpha]_D$ *c*, 1·053 in water
Residue A ..	.. 23·9	16·8	11·4	27·0	39·3		
Precipitate B	.. 4·5	30·1	10·9	4·9	7·1		
Fucoidin C ..	.. 6·4	29·5	42·5	26·9	39·2	26·2	— 121°

The formation of this insoluble residue A has led to a serious loss (27%) of fucose, although the resulting fucoidin C is purer than that recorded in Table III and almost completely free from colour. This resin-like compound A was found to be insoluble in organic solvents (benzene, carbon tetrachloride, acetic anhydride, acetone and ethyl acetate), and only slightly soluble in boiling concentrated hydrochloric acid and 40% sodium hydroxide. It contained Kjeldahl-N, 1·17, and alginic acid, 1·6% [standard precipitation method of Cameron, Ross & Percival (1948)].

When the combined centrifugates and washings from Expt. 7 (Table II), to which 40% formaldehyde (1·0 ml.) had also been added, were neutralized with sodium hydroxide, evaporated *in vacuo* to dryness, and treated as described for Expt. 12, similar results (shown in Table V) were obtained.

Table V

Fraction	Wt. as % of weed	Ash, %	Fucose, %	Fucose as % of total fucose	Fucose as % of fucose extracted
Residue A ..	.. 18·6	16·4	12·7	23·4	28·7
Precipitate B	.. 1·6	35·9	—	—	—
Fucoidin C ..	.. 12·1	37·4	32·2	38·5	47·2

That the formation of this insoluble residue was due to some complex with formaldehyde was shown as follows : Dried milled *F. vesiculosus* [Table I (4) ; 20·48 g.] was stirred with 0·17N-hydrochloric acid (200 ml.) at 70° for 1 hour, and the weed residue was centrifuged and washed with water (2 × 40 ml.). The centrifugate and washings (236 ml.) were divided into

two. One portion (118 ml.) was neutralized with sodium hydroxide, evaporated *in vacuo* at 50° to dryness (5·25 g.) and taken up in boiling water (100 ml.). When this dark-brown solution was centrifuged, only a very small residue was isolated (0·18% of the weed). The second portion (118 ml.) was stirred again at 70° for 1 hour, neutralized with sodium hydroxide and treated in the same way as the first portion. The insoluble residue amounted to 0·78% of the weed, thereby indicating that very little insoluble material is formed after 1 and 2 hours heating in the absence of formaldehyde.

When, however, the dark-brown centrifugates from these two experiments were combined, 40% formaldehyde (0·5 ml.) added, and the liquid was evaporated to dryness and taken up again in boiling water (100 ml.), an insoluble residue amounting to 12·8% of the weed was obtained (Found: ash, 13·0%). The solution after the separation of this residue was almost colourless.

Preparation of crude fucoidin from different species

Fucoidin was isolated from *P. canaliculata* [Table I (1)], *F. vesiculosus* [Table I (4)] and *A. nodosum* [Table I (2)], by the following general procedure: The dried milled weed (20 g.) was extracted three times with hydrochloric acid (200 ml.) at 70° for 1 hour at pH 2·0–2·5, as described for Expt. 7 (Table II), in the absence of formaldehyde. The normality of hydrochloric acid required to bring the pH within the range 2·0–2·5 at the first extraction varied slightly with different species, but generally lay between 0·10 and 0·17N. The combined centrifugates and washings were neutralized with sodium hydroxide, evaporated *in vacuo* at 50° to dryness, redissolved in water (125 ml.), treated with alcohol (54 ml.) to 30% (v/v) concentration, and precipitate B isolated as a brown powder. The centrifugate was then treated with alcohol (134 ml.) to 60% (v/v) concentration, and the crude fucoidin C isolated as a sandy-coloured solid. The analysis of the various fractions is shown in Table VI.

With *L. cloustoni* [Table I (5)], the frond (50·86 g.) was first stirred in the cold for 10 minutes with 0·09N-hydrochloric acid (500 ml.) to remove the bulk of the laminarin, centrifuged, and washed with water (2 × 100 ml.). This treatment was found to remove 31·7% of the total fucoidin. The frond residue was then treated with water to 400 ml., the pH reduced to 2·3 with 10N-hydrochloric acid (2·0 ml.), and the mixture stirred at 70° for 1 hour to extract the fucoidin. After two further extractions at 70° for 1 hour at pH 2·2, the combined centrifugates and washings from the 70° extractions were neutralized, evaporated to dryness, and treated exactly as described for the Fucaceae.

Table VI

Fraction	Species	Wt. as % of weed	Ash, %	Fucose, %	Fucose as % of total fucose	$[\alpha]_D$ in water
Weed Residue A	*P. canaliculata*	42·2	4·1	4·32	16·3	
	F. vesiculosus	42·9	2·6	4·81	20·4	
	A. nodosum	43·5	2·3	3·18	15·4	
	L. cloustoni frond	27·5	4·8	3·43	30·0	
Precipitate B	*P. canaliculata*	10·1	26·5	10·2	9·2	
	F. vesiculosus	6·5	32·7	11·8	7·6	
	A. nodosum	13·3	28·9	13·1	19·4	
	L. cloustoni frond	2·0	27·3	12·2	7·6	
Fucoidin C	*P. canaliculata*	25·5	30·1	33·3	75·9	− 110° (*c*, 0·908)
	F. vesiculosus	17·2	30·0	36·4	61·9	− 98° (*c*, 0·44)
	A. nodosum	15·7	28·7	30·7	53·5	− 115° (*c*, 0·910)
	L. cloustoni frond	1·9	31·5	33·3	20·2	− 106° (*c*, 0·452)

The results show that crude fucoidin, containing more than 30% fucose, can readily be prepared by fractional precipitation, with yields for the Fucaceae exceeding 50%. The poor yield (20·2%) in the case of *L. cloustoni* frond is due partly to the low initial fucose contents of the Laminarias, and partly to the loss (31·7%) of fucoidin in the initial laminarin extraction.

Purification of crude fucoidin

(1) *Reprecipitation with alcohol.*—Crude fucoidin (0·886 g.; fucose, 33·3; ash, 30·1%) was dissolved in water (9 ml.), alcohol (3·9 ml.) was added to 30% (v/v) concentration, but no precipitate formed. Alcohol (17·1 ml.) was then added to 70% (v/v), when the fucoidin appeared as a semi-colloid. This was coagulated immediately on adding sodium chloride (0·1 g. in 1 ml. water). The sticky precipitate was centrifuged, washed with alcohol and ether, and dried to a sandy-coloured powder (0·818 g.). (Found: ash, 29·8; fucose, 38·7%.)

Although the fucose content has been increased, reprecipitation does not remove the colour.

(2) *Precipitation with lead acetate and barium hydroxide.*—This method was used by Percival & Ross (1950) in their preparation of fucoidin. Crude fucoidin (1·803 g.; fucose, 33·3; ash, 30·1%) was dissolved in water (20 ml.), and lead acetate solution (2·0 g. $PbAc_2,3H_2O$ in 10 ml. water) added, but no precipitate formed. Cold saturated barium hydroxide (30 ml.) was then added until the solution was just pink to phenolphthalein. The lead hydroxide–fucoidin complex was centrifuged, washed with water (2 × 20 ml.), and decomposed by suspending in water (50 ml.) containing 4N-sulphuric acid (10 ml.), stirring for 3 hours, and leaving overnight. The lead sulphate was centrifuged, washed with hot water (2 × 20 ml.), and the centrifugate and washings were dialysed against tap-water until free from acid (3 days). The solution was then evaporated *in vacuo* at 50° to 20 ml., a small precipitate centrifuged, the solution treated with sodium chloride (0·1 g.) and alcohol to 70% (v/v) concentration, and the fucoidin was isolated as a brown powder (1·251 g.). (Found: ash, 24·6; fucose, 35·5%; i.e. 74·0% of the fucose in the crude product.)

This treatment, although it effects a slight purification, leads to considerable loss of fucoidin.

(3) *Treatment with formaldehyde.*—Crude fucoidin (1·460 g.; fucose, 36·4; ash, 30·0%; Table VI) was dissolved in water (50 ml.), 40% formaldehyde (0·50 ml.) added, and the solution evaporated at 50°/20 mm. to dryness. The dark-brown glass was extracted with hot water (20 ml.), and the insoluble residue A was centrifuged, washed with hot water (2 × 10 ml.) alcohol and ether, and dried to a brown powder. The light-brown centrifugate and water washings were treated with sodium chloride (0·1 g.) and alcohol to 70% (v/v) concentration, and fucoidin B isolated as an almost white powder. The analysis of A and B is recorded in Table VII.

Table VII

Fraction	Wt. as % of crude fucoidin	Ash, %	Fucose, %	Fucose as % of total fucose in crude fucoidin	Total sulphate	$[\alpha]_D$ in water (*c*, 1·06)
Residue A	.. 29·2	20·3	26·8	21·5		
Fucoidin B	.. 62·7	31·1	44·1	76·0	26·3	− 123°

The formation of this insoluble residue has therefore effected considerable purification, although it also leads to a 21·5% loss of fucoidin. This fucoidin B compares favourably in fucose content with the purest sample isolated by Percival & Ross (1950). The ash, however, is higher than that reported by these workers, although the total sulphate is lower. Expressed as a percentage of total fucose in the original *F. vesiculosus*, the yield of fucoidin B is 47·0%.

Large-scale preparation of fucoidin from *P. canaliculata*

The methods previously worked out on the small scale for preparing crude fucoidin and purifying the crude product have been used for the preparation of a quantity of fucoidin from *P. canaliculata* [Table I (1)]. The weed (200·4 g.) was extracted three times with hydrochloric acid (2 l.) at 60–70° for 1 hour at pH 2·1–2·2, exactly as described for Expt. 7 (Table II), except that the washing with water after each extraction was omitted. The combined centrifugates were neutralized with sodium hydroxide, evaporated *in vacuo* at 50°, and the extract dried for several hours at 50°/10 mm. Toluene was used as preservative, when it was necessary to leave the solution overnight during evaporation. The extract was dissolved in water (1250 ml.), treated with alcohol (540 ml.) to 30% (v/v) concentration, and precipitate B isolated as a brown powder. The centrifugate was then treated with alcohol (1340 ml.) to 60% (v/v), and crude fucoidin C isolated as a sandy-coloured solid (51·1 g.).

The crude product C (50·25 g.) was redissolved in water (500 ml.), 40% formaldehyde (17·5 ml.) added, the solution evaporated *in vacuo* at 50°, and the glass dried for several hours at 55°/10 mm. The glass was extracted with hot water (700 ml.), the insoluble residue D centrifuged, washed with hot water (300 ml.), alcohol and ether, and dried to a chocolate-coloured solid (13·54 g.). The centrifugate, with the water and alcohol washings, was treated with sodium chloride (3·0 g.) and further alcohol added to 70% (v/v) concentration. The fucoidin E, which was precipitated as a toffee-like solid, was centrifuged, washed with alcohol and ether, dried and ground to a light-brown powder (33·96 g.). The analysis of these fractions is given in Table VIII.

When fucoidin E (1·499 g.) was dissolved in water, and treated a second time with 40% formaldehyde (0·50 ml.) as described above, no insoluble residue was obtained, and the fucoidin was recovered unchanged in 95·9% yield on precipitation with alcohol.

Table VIII

Fraction	Wt. as % of weed	Ash, %	Fucose, %	Fucose as % of total fucose in weed
Precipitate B ..	.. 6·25	25·6	10·4	5·8
Crude fucoidin C	.. 25·5	30·7	35·7	81·3
Residue D ..	.. 6·9	24·1	26·2	16·1
Fucoidin E ..	.. 17·2	32·0	41·1	63·2

Summary

Methods for the extraction and isolation of fucoidin from brown marine algae have been worked out on the laboratory scale.

Optimum conditions for the extraction of fucoidin consist of stirring for one hour at 70° one part (by weight) of the dried milled weed (all passing 64 mesh) with ten parts (by volume) of hydrochloric acid at pH 2·0–2·5. This treatment removes about 50% of the fucoidin, whereas three acid extractions remove more than 80%. Fucoidin can also be extracted by heating one part of the dried milled weed with ten parts of water at 100° for 3 to 7½ hours. This removes 55–60% of the fucoidin, but more efficient extractions can be obtained by increasing the water : weed ratio, the extraction time or the number of extractions. Aqueous extractions, however, are not recommended because of the difficulty of separating the weed residue from the solution.

Crude fucoidin is isolated from the acid extracts by neutralization and evaporation to dryness, solution in water, and fractional precipitation with alcohol at 30 and 60% (v/v) concentration. The 60% fraction is crude fucoidin containing 30–36% fucose (as $C_6H_{12}O_5$). This crude product has been isolated in 76% yield from *P. canaliculata*, 62% from *F. vesiculosus*, 53% from *A. nodosum* and 20% from *L. cloustoni* frond. Fucoidin, containing more than 40% fucose, can be prepared from the crude product by treating with formaldehyde, and separating the insoluble compound formed. In this way fucoidin, containing fucose (as $C_6H_{12}O_5$), 44·1 ; ash, 31·1 ; total sulphate, 26·3% ; and $[\alpha]_D$—123° in water, has been isolated from *F. vesiculosus* in 47% yield.

In a larger scale experiment fucoidin, containing 41·1% fucose, has been prepared from 200 g. of *P. canaliculata* in 63% yield.

Acknowledgment

The work described in this paper forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association, to whom the authors are indebted for permission to publish.

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Received 19 October, 1951

References

Bird, G. M. & Haas, P. (1931). *Biochem. J.* **25**, 403.

Black, W. A. P. (1948). *J. Soc. chem. Ind.* **67**, 165, 169, 172.

Black, W. A. P., Cornhill, W. J., Dewar, E. T., Percival, E. G. V. & Ross, A. G. (1950). *J. Soc. chem. Ind.* **69**, 317.

Black, W. A. P., Cornhill, W. J., Dewar, E. T. & Woodward, F. N. (1951). *J. appl. Chem.* **1**, 505.

Bray, H. G., Henry, H. & Stacey, M. (1946). *Biochem. J.* **40**, 124.

Cameron, M. C., Ross, A. G. & Percival, E. G. V. (1948). *J. Soc. chem. Ind.* **67**, 161.

Conchie, J. & Percival, E. G. V. (1950). *J. chem. Soc.* p. 827.

Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1948). *J. chem. Soc.* p. 1679.

Folkes, B. F., Grant, R. A. & Jones, J. K. N. (1950). *J. chem. Soc.* p. 2136.

Hoagland, D. R. & Lieb, L. L. (1915). *J. biol. Chem.* **23**, 287.

Hockett, R. C., Phelps, F. P. & Hudson, C. S. (1939). *J. Amer. chem. Soc.* **61**, 1658.

James, S. P. & Smith, F. (1945). *J. chem. Soc.* p. 739.

Kylin, H. (1913). *Z. physiol. Chem.* **83**, 171.

Kylin, H. (1915). *Z. physiol. Chem.* **94**, 337.

Lunde, G., Heen, E. & Öy, E. (1937). *Z. physiol. Chem.* **247**, 189.

Nelson, W. L. & Cretcher, L. H. (1931). *J. biol. Chem.* **94**, 147.

Percival, E. G. V. & Ross, A. G. (1950). *J. chem. Soc.* p. 717.

Vasseur, E. (1948). *Acta. chem. scand.* **2**, 900.

Printed in Great Britain by Butler & Tanner Ltd., Frome and London

MANUFACTURE OF ALGAL CHEMICALS. V.*—Laboratory-scale Isolation of D-Glucose from Laminarin

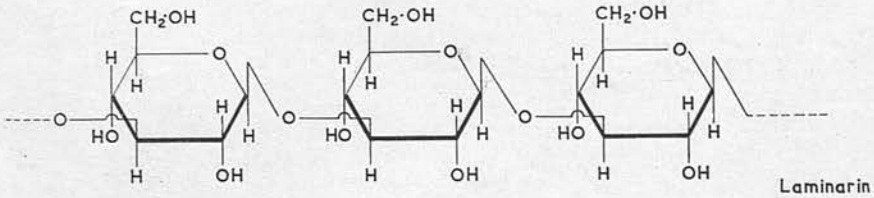
By W. A. P. BLACK, E. T. DEWAR AND F. N. WOODWARD

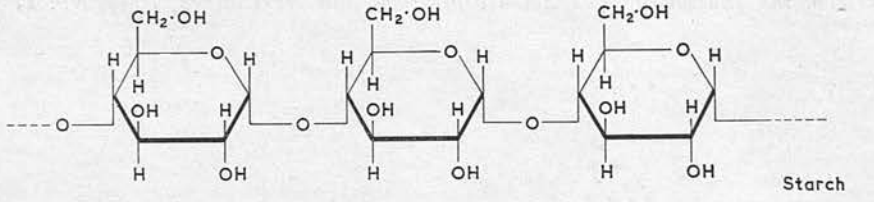
A method for the isolation of D-glucose from laminarin has been worked out on the laboratory scale, with a view to the ultimate development of a process suitable for large-scale production. Laminarin is hydrolysed quantitatively to D-glucose by heating an 18% (w/w) solution of the polysaccharide in 0·05N-hydrochloric acid at 135° in an autoclave for one hour. The resulting hydrolysate is neutralized and decolorized, and the glucose is crystallized from water as the monohydrate.

The production of glucose from laminarin is closely related to the commercial production of crystalline dextrose by the acid hydrolysis of starch.

Introduction

Laminarin is a glucose polysaccharide occurring exclusively in brown marine algae. It is a glucan containing β-D-glucopyranose units linked through the 1 : 3-positions,[1, 2, 3] and thereby differing fundamentally from the starch of land plants, in which the repeating units are α-D-glucopyranose residues linked through carbon atoms 1 and 4.





Laminarin isolated from *Laminaria cloustoni* frond is insoluble in cold water, whereas that isolated from *L. digitata* frond and many other species is water-soluble in the cold, and the preparation of these forms has recently been investigated in some detail.[4] Studies by Connell, Hirst & Percival[2] and Percival & Ross[3] have failed to detect any major chemical differences in the two forms, methylation and hydrolysis giving a yield of tetramethyl glucose corresponding to a chain length of about 20 glucose units in each case.

The conversion of laminarin to glucose by treatment with acids has been investigated by numerous workers. Kylin[5] found that laminarin from *L. cloustoni*, in solution (1·6%) in 5% sulphuric acid, was hydrolysed completely on heating at 100° for 4 hours, a 90% conversion to glucose being determined polarimetrically and by reducing power towards Fehling's solution. He also showed[6] that laminarin from *Desmarestia aculeata* gave 90% of glucose on heating a 2·0% solution in 3% sulphuric acid on the boiling-water bath for 4 hours. Gruzewska[7] carried out a number of experiments to determine the optimum conditions of hydrolysis of laminarin both by acids and by diastases. She found that laminarin from *L. flexicaulis* gave a 97% conversion to glucose on heating a 0·45% solution in 6% hydrochloric acid in an autoclave at 120° for 30 minutes. The hydrolysate, on neutralization, concentration and treatment with phenylhydrazine, yielded the characteristic crystals of glucosazone. Colin & Ricard[8] obtained glucose in crystalline condition from the hydrolysis of laminarin (from *L. flexicaulis*) with 5% sulphuric acid at 120° in an autoclave for 30 minutes. They carried out a series of hydrolyses with 5% sulphuric acid and 2% hydrochloric acid at 120° for various times, the course of the reactions being followed polarimetrically and by reducing power with Fehling's solution. The specific rotations of the hydrolysates varied from 48·8 to 53·0°, but were generally slightly less than that of pure glucose.[9] Barry[10] hydrolysed laminarin with 5% sulphuric acid on the boiling-

* Part IV : *J. Sci. Fd Agric.*, 1952, **3**, 122 ; Part III : *J. appl. Chem.*, 1951, **1**, 505 ; Part II : *J. appl. Chem.*, 1951, **1**, 414

water bath to constant rotation, but the glucose estimated polarimetrically never exceeded 90% of the yield calculated from the equation:

$$(C_6H_{10}O_5)_n + nH_2O = nC_6H_{12}O_6$$

With N-hydrochloric acid at 100° for 2½ hours, however, a 98·5% conversion to glucose was obtained.

Barry[11] isolated the disaccharide, laminaribiose, by heating laminarin with N-oxalic acid until the hydrolysis was about two-thirds complete (7 hours, 100°), neutralizing and destroying the glucose formed with yeast. The optically active liquid remaining was dissolved in methanol, and the laminaribiose was obtained as a powder by fractional precipitation with ethanol and ether. Recently, Connell, Hirst & Percival[2] have obtained the disaccharide by partial hydrolysis of laminarin with oxalic acid, and separation of the sugars on a cellulose column.

Proof of the structure of laminaribiose as 3-β-D-glucosyl D-glucose has been obtained by its synthesis from 1 : 2–5 : 6-di*iso*propylidene glucose and tetra-acetyl glucosyl bromide.[12, 13]

Cameron, Ross & Percival[14] found that laminarin from *L. cloustoni* in 1% solution was completely hydrolysed with N-sulphuric acid at 100° in 4½ hours. Connell, Hirst & Percival[2] showed that hydrolysis at 95° in 0·85N-hydrochloric acid was complete in 2½ hours, giving glucose, 96% (polarimetric estimation) and 95% (hypoiodite). No sugar other than glucose could be detected on the paper chromatogram. Soluble laminarin from *L. digitata* was completely hydrolysed with N-sulphuric acid at 95° after 4½ hours to give a 95·3% conversion to glucose.[3]

The hydrolysis of laminarin by enzymes has also been studied by various workers, although the early results are somewhat conflicting. Torup[15] stated that laminarin was not attacked by ptyalin, nor by diastase from pancreas or malt. Kylin[5] claimed that the polysaccharide was slowly hydrolysed by malt diastase, but he has since withdrawn this claim;[16] in this more recent work, malt diastase, takadiastase, ptyalin and emulsin were found to be without action, and the laminarin was precipitated unchanged after three days at 33°. Gruzewska[7] showed that the action of various animal and vegetable diastases was very slow, but that the juice of the snail, *Helix pomata*, was very effective, the hydrolysis to glucose being almost complete in 1 hour. This was confirmed by Colin & Ricard[8] and by Barry;[11] Barry found that a glycerol extract of the limpet (*Patella*) was also capable of hydrolysing laminarin to glucose.

Dillon & O'Colla[17] found that wheat β-amylase slowly hydrolyses laminarin, yielding glucose (70%) and a disaccharide after several days. Since malt diastase was inactive, they attributed the action to some factor ('laminarinase') other than the small amount of α-amylase in the crude wheat enzyme. The occurrence of a similar laminarinase has since been demonstrated in extracts of oats, barley, potato and hyacinth bulbs.[18] Recently, Peat, Thomas & Whelan,[19] in their studies on the enzymic synthesis and degradation of starch, have isolated a Z-enzyme from the soya bean, the apparent function of which is to supplement the action of β-amylase or phosphorylase on the amylase fraction of starch. They found that stock soya β-amylase, which still contained the Z-enzyme, hydrolysed laminarin, the limiting yield of glucose (72·3% after 142 hours) agreeing closely with the value of 70% reported by Dillon & O'Colla[17] for wheat laminarinase. There is thus a strong indication that the Z-enzyme of soya bean and the laminarinase of wheat are identical. Dillon & O'Colla also showed that laminarin is slowly attacked by emulsin (72·8% conversion to glucose after 46 days at 35°), but is not hydrolysed by pure crystalline β-amylase nor by freeze-dried salivary α-amylase.

In the present investigation, the complete hydrolysis of laminarin with acids has been studied under various conditions to determine the optimum conditions for the production and isolation of glucose from this polysaccharide. Commercial glucose (dextrose) is manufactured by the acid hydrolysis of starch, chiefly corn starch at the present time. The usual practice is to suspend the starch in water at a concentration of 15–20%, add hydrochloric acid until the normality is about 0·03, and then hydrolyse in an autoclave at 150° for about 30 minutes. The converted liquor is neutralized with sodium carbonate, passed through skimming tanks to remove floating impurities, and decolorized with bone char and activated carbon. The liquor is finally evaporated to a syrup containing 75–78% dry matter, and crystallized slowly over a period of days at about 41°. The crystalline mass of dextrose monohydrate (α-D-glucose monohydrate) is centrifuged, washed with water and dried in a stream of air. The commercial production of crystalline dextrose has recently been reviewed by Dean & Gottfried.[20]

Experimental; discussion of results

The laminarin used in these investigations was the insoluble form extracted from *L. cloustoni* frond. It was 'recrystallized' twice from water, and contained ash, 0·7%; $[\alpha]_D^{16}$ — 12·7° in water (*c*, 2·134). The pure glucose used was AnalaR anhydrous α-D-glucose, which gave

$[\alpha]_D^{14}$ + 107·6° (5 minutes) → + 52·4° in water (*c*, 4·165). Pigman & Goepp[21] quote $[\alpha]_D^{20}$ + 112·2° → + 52·7° in water (*c*, 4) for pure α-D-glucose. All rotations were measured in a 2-dm. tube.

Rate of hydrolysis of laminarin with acids

The rate of liberation of glucose from laminarin was followed by estimating the reducing power in the hydrolysate by Shaffer & Somogyi's method.[22] In Expts. 1–8 (Table I), the polysaccharide was heated under reflux on a boiling-water bath with the appropriate acid, and 2-ml. portions of the hydrolysate were withdrawn at intervals and allowed to cool. Portions of solution (1 ml.) were then transferred by pipette to a standard flask (250 ml.), water (20–30 ml.) was added, the solution neutralized with 0·1N-sodium hydroxide, using one drop of phenol red as indicator, and made up to the mark. The reducing power of 5-ml. portions of this solution was then estimated with Shaffer and Somogyi reagent 50 against a 5-ml. water blank as described by Cameron, Ross & Percival:[14]

$$\% \text{ Conversion to glucose} = \frac{\text{Titration difference} \times 250 \times 25 \times 162{\cdot}1 \times 26{\cdot}7 \times 100}{9{\cdot}02 \times 5 \times 1 \times 180{\cdot}2 \times 25 \times (\text{wt. of polysaccharide}) \times 1000}$$

The factor of 26·7/25 was introduced to allow for the increase in volume on solution. When anhydrous glucose (2·749 g.) was dissolved in exactly 25 ml. of N-sulphuric acid, the resulting volume was 26·7 ml.; roughly the same increase was observed with the other acids used. Laminarin (2·480 g.) on hydrolysis should yield 2·757 g. of glucose according to the equation:

$$(C_6H_{10}O_5)_n + nH_2O = nC_6H_{12}O_6$$

Two experiments (Expts. 2 and 5) were carried out with potato starch in order to compare the relative rates of hydrolysis of laminarin and starch.

In Expts. 7 and 8, the laminarin was dissolved in the hydrochloric acid in a Lintner pressure-bottle with a rubber stopper, which was enclosed in a metal frame to prevent the stopper being blown out, and the hydrolysis was carried out at 135° in a Pentecon autoclave (gauge pressure, 33 lb./sq. in.). The period of hydrolysis was taken as the time between the thermometer reaching 135° and the pressure being released, the times taken to reach pressure (about 15 minutes) and release pressure (about 5 minutes) being neglected. The technique was slightly altered in Expt. 9, where the concentration of polysaccharide, normality of acid and temperature of hydrolysis are similar to those employed in the commercial production of glucose from starch. The laminarin was dissolved in a known weight of 0·05N-hydrochloric acid, and the solution hydrolysed at 135°. Portions (2 ml.) were withdrawn at intervals and allowed to cool, 1 ml. was transferred by pipette to a weighed beaker, the weighed solution made up to 500 ml., and the reducing power determined as before on 5 ml.

The results in Table I show that laminarin is hydrolysed to glucose only slightly less rapidly than potato starch. Hydrochloric acid is to be preferred to sulphuric acid, 0·29N-hydrochloric acid (Expt. 6) being more efficient than 0·5N-sulphuric acid (Expt. 3). Expt. 9 indicates that the conditions used commercially for the hydrolysis of starch will also apply to laminarin, with the exception of the time of conversion, which may have to be increased slightly for laminarin.

Specific rotation of laminarin on hydrolysis

As a check on the rate of liberation of glucose determined by reducing power, the hydrolysis of laminarin was followed polarimetrically. Laminarin (3·600 g.) was dissolved in 0·057N-hydrochloric acid (16·86 g.) to give a 17·60% (w/w) laminarin solution (i.e. 19·57% as glucose, $C_6H_{12}O_6$), and the solution was hydrolysed at 135° as in Expt. 9 (Table I). Portions (5 ml.) were withdrawn at intervals, weighed, filtered, made up to 50 ml. in Expts. 1 and 2, and to 200 ml. in Expt. 3, and the rotations measured in a 2-dm. tube. The percentage conversion to glucose was calculated assuming the $[\alpha]_D^{20}$ of D-glucose at equilibrium to be + 52·7°.[21] The results are recorded in Table II.

These results are in good agreement with Expt. 9 (Table I) and with the figures quoted by Colin & Ricard.[8]

Rate of destruction of glucose by N-sulphuric acid

There are several references in the literature to the destruction of glucose during the determination of starch by hydrolysis with acids, and factors have been introduced to compensate for these losses.[23–25] Since most of the results in the literature concern rather dilute solutions, it was decided to investigate the degrading effect of acid in a glucose solution similar to those

formed in Table I. Anhydrous glucose (2·758 g., equivalent to 2·482 g. as $C_6H_{10}O_5$) was heated under reflux at 100° with N-sulphuric acid (25 ml.), and the reducing power determined at intervals as described previously. The results are shown in Table III. When the results after 7·5 hours in Expts. 1 and 2 (Table I) are corrected for a 5% destruction of glucose, the percentage conversions for laminarin and starch are increased to 96·2 and 99·4 respectively.

Isolation of β-penta-acetyl glucose from laminarin

In an attempt to show that glucose could be isolated quantitatively from laminarin on hydrolysis, it was decided to prepare the β-penta-acetate which is almost insoluble in water. Gruzewska[7] and Barry[10] confirmed the presence of glucose in laminarin by preparing glucosazone, but their experiments were not quantitative. Colin & Ricard[8] state that they obtained glucose in crystalline condition but give no details of the method used nor yield obtained. Laminarin (4·524 g.) was hydrolysed at 100° with N-sulphuric acid (50 ml.) for 5 hours with occasional stirring, the hydrolysate was centrifuged to remove a small brown residue, diluted to about 100 ml., passed through a Zeo-Karb 225 column to remove any cations derived from the ash, and the effluent neutralized by adding Amberlite IR-4B-OH (40 ml.) and stirring for about 10 minutes. The resin was filtered off, washed thoroughly with water, and the filtrate and washings evaporated *in vacuo* at 50° to a yellow syrup, which was dissolved in boiling methanol (25 ml.) and the solution centrifuged. The centrifugate was evaporated to dryness, and the resulting syrup dried *in vacuo* over phosphorus pentoxide (4·993 g.).

Acetylation was carried out with acetic anhydride and sodium acetate.[26] The syrup was heated with powdered anhydrous sodium acetate (2·5 g.) and acetic anhydride (25 ml.) at 100° with frequent shaking until completely dissolved, and then heated for a further 2 hours. The brown solution was poured into ice-cold water (250 ml.), when the viscous oil solidified almost immediately. The acetate was filtered, washed thoroughly with water, and dried *in vacuo* over phosphorus pentoxide (6·974 g.). Yield, 64·0%. On recrystallization from 67% ethanol, the yield dropped to 49·6%. M.p. 128–129°, not depressed on admixture with authentic β-penta-acetyl glucose (m.p. 129–130°); $[\alpha]_D^{15}$ + 7·9° (*c*, 6·11 in glacial acetic acid). Hudson & Dale[27] quote m.p. 132° and $[\alpha]_D^{20}$ + 3·7° (*c*, 6·267 in glacial acetic acid) for pure β-penta-acetyl glucose.

When potato starch (4·530 g.) was hydrolysed and acetylated under identical conditions, the yield of crude acetate was 67·6%, which is not significantly different from laminarin. With pure glucose (5·005 g.), however, the crude acetate was formed in 84·2% yield, which fell to 70·6% on recrystallization from 67% ethanol. It is evident that the small amount of impurity in hydrolysed laminarin or starch seriously lowers the yield of this compound.

Crystallization of glucose from various solvents

Before attempting to isolate glucose itself from laminarin hydrolysates, preliminary experiments were carried out to find the most suitable conditions for crystallizing small amounts of glucose on the laboratory scale in relatively quantitative yield.

Water.—Glucose is very soluble in water, 100 g. of saturated solution containing 45·0 g. of glucose at 15° and 84·9 g. at 91°.[28] When glucose (27·6 g.) was dissolved in hot water (10 ml.) to give a 73·4% (w/w) solution and the syrup placed in the refrigerator at 5°, crystals began to form after 3 days. After 7 days, the crystalline material was centrifuged, washed with water (5 ml.), ethanol (2 × 25 ml.) and ether (25 ml.), and dried in the atmosphere (9·65 g.). Yield, 35·0%; $[\alpha]_D^{16}$ + 101·3° (5 minutes) → + 52·4° in water (*c*, 4·15). The crystals were therefore anhydrous, and chiefly the α-form. In view of the high solubility of glucose in water, this solvent was abandoned for small-scale quantitative work.

When glucose is crystallized from concentrated aqueous solutions at 40° in the presence of a large amount of seed crystals, the monohydrate is formed.[20]

Methanol.—Glucose is relatively soluble in hot methanol. Methanol (100 g.) dissolves 1·5 g. of glucose at 0° and 11·1 g. at 76°.[29] Glucose (27·5 g.) was dissolved in water and evaporated *in vacuo* to a syrup, which was dried *in vacuo* over phosphorus pentoxide. The syrup was dissolved by refluxing with methanol (150 ml.), and the solution cooled, seeded with glucose, and kept at 5° for 3 days with occasional stirring. Crystallization was slow, particularly during the first day. The crystalline glucose was filtered, washed with ethanol and ether, and dried at 100° for 45 minutes (23·22 g.). Yield, 84·5%. The crystals were an αβ-mixture: $[\alpha]_D^{16}$ +62·1° (6 minutes) → +52·6° in water (*c*, 4·214).

When a syrup (28·1 g.), obtained by the hydrolysis of laminarin (24·75 g.) with N-H_2SO_4 (250 ml.) as described for the preparation of β-penta-acetyl glucose, was dissolved in methanol (150 ml.), seeded and kept at 5° for 4 days, glucose was isolated as a white crystalline powder

Table I

Rate of hydrolysis of laminarin and starch with acids

Expt. No.	Polysaccharide hydrolysed, g.	Acid used *	Concn. of polysaccharide (w/w), %	Temp. of hydrolysis, °C.	% conversion to glucose after time (hr.) 0·5	1·0	1·5	2·0	3·0	5·0	6·0	7·5
1 ..	Laminarin, 2·478	N-H_2SO_4	9·0	100	29·4	58·0	—	85·3	91·1	92·7	—	91·4
2 ..	Starch, 2·480	N-H_2SO_4	9·0	100	40·0	71·7	—	91·3	93·6	95·2	—	94·3
3 ..	Laminarin, 2·484	0·5N-H_2SO_4	9·0	100	11·4	19·9	—	42·2	59·5	82·8	—	92·5
4 ..	,, 2·485	0·25N-H_2SO_4	9·0	100	5·4	—	—	21·7	30·8	52·2	61·7	70·4
5 ..	Starch, 2·485	0·25N-H_2SO_4	9·0	100	8·1	—	—	36·0	50·3	71·5	79·9	85·1
6 ..	Laminarin, 2·485	0·29N-HCl	9·0	100	14·9	30·9	—	59·9	77·5	91·5	92·5	93·9
7 ..	,, 2·487	0·1N-HCl	9·0	135	92·8	94·2	—					
8 ..	,, 2·483	0·05N-HCl	9·0	135	90·4	98·2	95·7					
9 ..	,, 3·599	0·05N-HCl	17·62	135	83·3	92·9	92·0					

* 25 ml. used for Expts. 1–8; 16·83 g. for Expt. 9

Table II

Specific rotation of laminarin on hydrolysis

Expt. No.	Time of hydrolysis, hr.	Concn., g. of $C_6H_{12}O_6$/100 ml.	$[\alpha]_D^{17}$	Conversion to glucose, %
1 ..	0·5	2·090	+ 49·3	93·5
2 ..	1·0	2·086	+ 49·8	94·6
3 ..	1·5	0·523	+ 49·7	94·3

Table III

Action of N-sulphuric acid on glucose

Time of heating, hr.	0·0	1·0	2·0	3·0	7·0
Glucose in soln., %	100·3	98·6	97·7	97·1	95·0

Table IV

Crystalline glucose fractions from hydrolysed laminarin

Fraction	Crystallization time at 5°, days	Equilibrium $[\alpha]_D^{17}$ in water (*c*, 4·0)	Purity (calc. from rotation), %	Yield, %
Glucose A ..	7	+ 52·5°	99·6	24·3
,, B ..	7	+ 51·2°	97·1	30·1
,, C ..	7	+ 51·4°	97·6	8·9
,, D ..	18	+ 50·1°	95·1	8·1
,, E ..	31	+ 53·0°		4·4

Table V

Crystalline glucose fractions from hydrolysed laminarin

Fraction	Moisture, %	Dry wt. (corr. for added seed), g.	Yield, %	$[\alpha]_D^{20}$ (5 min.) in water (*c*, 3·7 on anhyd. basis)	Equilibrium $[\alpha]_D^{20}$ in water	Purity (calc. from rotation), %
Glucose A ..	8·7	31·40	56·9	+ 103·4°	+ 52·5°	99·6
,, B ..	5·8	5·73	10·4	+ 94·2	+ 53·3	

(11·30 g.). Yield, 41·1%; $[\alpha]_D^{15}$ + 69·4° (6 minutes) → + 52·0° in water (*c*, 4·04). M.p. 145–146°.

Ethanol, 92% (*v/v*).—This is a very convenient medium for crystallizing small quantities of pure glucose (Dr. D. J. Bell, private communication). When glucose (4·998 g.) was dissolved in water (4 ml.) and ethanol (46 ml.), crystals formed almost immediately. After 2 hours at room temperature, and overnight at 5°, the glucose was isolated and dried (4·163 g.). Yield, 83·3%; $[\alpha]_D^{14}$ + 62·4° (6 minutes) → + 52·3° in water (*c*, 4·165). The product was again an $\alpha\beta$-mixture. When, however, this solvent was applied to laminarin hydrolysates, the solutions failed to crystallize and syrups were deposited. Reducing the concentration of sugar in the solution still failed to give a crystalline product.

From these experiments, methanol was considered to be the most suitable solvent for the small-scale, quantitative crystallization of glucose, although the crystallization rate was very slow.

Hydrolysis of laminarin and crystallization of glucose from methanol

The conditions used in Expt. 9 (Table I) were employed. Laminarin (49·67 g.) was dissolved in 0·057N-hydrochloric acid (220 ml.) to give an 18·0% (w/w) laminarin solution, and the solution hydrolysed at 135° for 1 hour in a flask plugged with cotton wool. The hydrolysate was centrifuged to remove a small brown precipitate, the centrifugate passed through a Zeo-Karb 225 column, and the effluent neutralized by adding Amberlite IR-4B-OH and stirring for about 10 minutes. The resin was filtered off, washed thoroughly with water, and the filtrate and washings evaporated *in vacuo* at 50° to a brown solution (300 ml.), which was decolorized with charcoal (25 g.), filtered, and the filtrate evaporated to a colourless syrup and dried *in vacuo* over phosphorus pentoxide. The syrup (56·1 g.) was dissolved by refluxing with methanol (300 ml.), the solution centrifuged to remove a small residue, the centrifugate seeded with α-D-glucose (2·00 g.) and kept at 5° for 7 days with occasional stirring. The crystalline glucose A was filtered, washed with ethanol and ether, and dried at 100° for 45 minutes to a pure white powder.

The mother liquor was evaporated to a syrup (40·1 g.), which was dissolved in 0·057N-hydrochloric acid (160 ml.) and the solution 'reconverted' at 135° for 1 hour. The hydrolysate was again neutralized, treated with charcoal (20 g.) and worked up to a syrup (37·6 g.), which was dissolved in methanol (150 ml.) and seeded (1·00 g.). After 7 days at 5°, the crystalline glucose B was isolated as before.

The mother liquor on evaporation was again hydrolysed with 0·057N-hydrochloric acid, and the solution worked up as described above to give crystalline glucose C. Two further crops of crystals were obtained by evaporating the mother liquor and crystallizing from small volumes of methanol. The analysis of these crystalline fractions is shown in Table IV.

The total yield of crystalline glucose therefore amounts to 75·8% of the theoretical yield. However, in view of the extremely slow rate of crystallization of glucose from methanol, an attempt has been made to crystallize from water using the conditions employed in the commercial production of dextrose from starch.[20]

Hydrolysis of laminarin and crystallization of glucose from water

Laminarin (49·67 g.) was dissolved in 0·057N-hydrochloric acid (220 ml.), and the solution hydrolysed, treated with ion-exchange resins, decolorized with charcoal and evaporated to dryness exactly as described in the previous experiment. The resulting colourless syrup was dissolved in water to give a solution containing 77·5% (w/w) dry matter, and the solution seeded with α-D-glucose (13·9 g.) and allowed to stand at room temperature (20°), when the viscous solution solidified overnight. After 2 days at room temperature, followed by 2 days at 5°, the crystalline glucose A was filtered, washed with ethanol and ether, and dried *in vacuo* over phosphorus pentoxide. The results in Table V show that this fraction is α-D-glucose monohydrate (Calc. for $C_6H_{12}O_6,H_2O$: H_2O, 9·1%).

The filtrate on evaporation gave a syrup (17·5 g.) which was dissolved in water to give a 76·1% dry matter content and seeded with α-D-glucose (4·4 g.). After 1 day at 20° and 1 day at 5°, the glucose B was isolated as before. This fraction was found to be a mixture of the monohydrate and the anhydrous material.

The analysis of these fractions is shown in Table V.

The yield of crystalline glucose therefore amounts to 67·3% of the theoretical yield after a crystallization time of only 6 days. The yield of 56·9% after the first crystallization is in good agreement with the figure of about 60% generally obtained for starch.[20] Greater efficiency could no doubt be obtained by crystallizing under the carefully controlled conditions of time

and temperature employed in the commercial production of dextrose, and also by a more thorough working up of the mother liquors.

Summary

Optimum conditions have been worked out for the complete conversion of laminarin to glucose. Hydrolysis is effected by heating an 18% (w/w) solution of the polysaccharide in 0·05N-hydrochloric acid at 135° in an autoclave for 1 hour, the reducing power and specific rotation of the hydrolysate indicating a 93–95% conversion to D-glucose. Laminarin is hydrolysed by acids only slightly less rapidly than potato starch.

β-Penta-acetyl glucose has been prepared from hydrolysed laminarin in 64·0% yield (crude product), which compares favourably with a yield of 67·6% from hydrolysed starch under the same conditions. Pure glucose, however, gives a much higher yield (84·2%) of crude acetate, indicating that impurities in the hydrolysates of laminarin and starch seriously lower the yield of this compound.

Methanol has been found to be a satisfactory solvent for crystallizing relatively small amounts of glucose, although solutions must be allowed to stand for long periods to obtain maximum yields of crystals. Pure glucose can be recrystallized from methanol in 84·5% yield after a crystallization time of 3 days at 5°. Much longer times are required for laminarin hydrolysates. In a hydrolysis using 50 g. of laminarin, crystalline glucose has been prepared in 75·8% yield. The glucose was obtained in 5 fractions with purities ranging from 99·6 to 95·1%.

When glucose is crystallized from concentrated aqueous solution at 5° in the absence of any seed, crystallization is slow and the yield is poor. When, however, the solution is treated with a large quantity of seed crystals, and allowed to stand at 20°, crystallization sets in rapidly and a good yield of α-D-glucose monohydrate is obtained. In a hydrolysis using 50 g. of laminarin, pure crystalline glucose has been isolated in 2 fractions in 67·3% yield, after a crystallization time of only 6 days. The yield of the first crop of crystals (56·9%) compares favourably with approximately 60% obtained in the commercial production of dextrose from starch.

Acknowledgment

The work described in this paper forms part of the programme of research and development on seaweed undertaken by the Institute of Seaweed Research, to whom the authors are indebted for permission to publish.

Institute of Seaweed Research
Inveresk
Midlothian

Received 3 September, 1952

References

[1] Barry, V. C., *Sci. Proc. R. Dublin Soc.*, 1939, **22,** 59
[2] Connell, J. J., Hirst, E. L. & Percival, E. G. V., *J. chem. Soc.*, 1950, p. 3494
[3] Percival, E. G. V. & Ross, A. G., *J. chem. Soc.*, 1951, p. 720
[4] Black, W. A. P., Cornhill, W. J., Dewar, E. T. & Woodward, F. N., *J. appl. Chem.*, 1951, **1,** 505
[5] Kylin, H., *Hoppe-Seyl. Z.*, 1915, **94,** 337
[6] Kylin, H., *Hoppe-Seyl. Z.*, 1918, **101,** 236
[7] Gruzewska, Z., *Bull. Soc. Chim. biol., Paris*, 1921, **3,** 490
[8] Colin, H. & Ricard, P., *Bull. Soc. Chim. biol., Paris*, 1930, **12,** 88
[9] Colin, H. & Ricard, P., *C. R. Acad. Sci., Paris*, 1929, **188,** 1449
[10] Barry, V. C., *Sci. Proc. R. Dublin Soc.*, 1938, **21,** 615
[11] Barry, V. C., *Sci. Proc. R. Dublin Soc.*, 1941, **22,** 423
[12] Freudenberg, K. & Oertzen, K., *Liebigs Ann.*, 1951, **574,** 37
[13] Bächli, P. & Percival, E. G. V., *J. chem. Soc.*, 1952, p. 1243
[14] Cameron, M. C., Ross, A. G. & Percival, E. G. V., *J. Soc. chem. Ind., Lond.*, 1948, **67,** 161
[15] Torup, S., *Pharmacia, Kristiania*, 1909, **6,** 153
[16] Kylin, H., *K. fysiogr. Sällsk. Lund Förh.*, 1944, **14,** 226
[17] Dillon, T. & O'Colla, P., *Nature, Lond.*, 1950, **166,** 67
[18] Dillon, T. & O'Colla, P., *Chem. & Ind.*, 1951, p. 111
[19] Peat, S., Thomas, G. J. & Whelan, W. J., *J. chem. Soc.*, 1952, p. 722
[20] Dean, G. R. & Gottfried, J. B., *Advanc. Carbohyd. Chem.*, 1950, **5,** 127
[21] Pigman, W. W. & Goepp, R. M., 'Chemistry of the Carbohydrates', 1948, p. 97 (New York: Academic Press Inc.)
[22] Shaffer, P. A. & Somogyi, M., *J. biol. Chem.*, 1933, **100,** 695
[23] Lampitt, L. H., Fuller, C. H. F. & Goldenberg, N., *J. Soc. chem. Ind., Lond.*, 1947, **66,** 117
[24] Lampitt, L. H., Fuller, C. H. F., Goldenberg, N. & Vine, M., *J. Sci. Fd Agric.*, 1950, **1,** 371
[25] Pirt, S. J. & Whelan, W. J., *J. Sci. Fd Agric.*, 1951, **2,** 224
[26] Fischer, E., *Ber. dtsch. chem. Ges.*, 1916, **49,** 584
[27] Hudson, C. S. & Dale, J. K., *J. Amer. chem. Soc.*, 1915, **37,** 1264
[28] Jackson, R. F. & Silsbee, C. G., *Bur. Stand., Sci. Papers*, No. 437, 1922, **17,** 715
[29] Gillis, J. & Nachtergaele, H. N., *Rec. Trav. chim. Pays-Bas*, 1934, **53,** 31

Printed in Great Britain by Butler & Tanner Ltd., Frome and London

Reprinted from the Journal of The Science of Food and Agriculture, 1953, No. 2, pp. 85–91.

MANUFACTURE OF ALGAL CHEMICALS. VI.—Laboratory-scale Isolation of L-Fucose from Brown Marine Algae

By W. A. P. BLACK, W. J. CORNHILL, E. T. DEWAR and F. N. WOODWARD

Methods for the isolation of crystalline L-fucose from both dried milled brown seaweed and fucoidin have been worked out on the laboratory scale, with a view to the ultimate development of a process suitable for large-scale production. Preparation from the original seaweed involves hydrolysis with acid, formation of fucose phenylhydrazone, and subsequent decomposition of the phenylhydrazone. The isolation from fucoidin involves hydrolysis of the polysaccharide by heating a 16% (w/w) solution in 0·25N-hydrochloric acid at 135° in an autoclave for two hours, removal of salts and acid with ion-exchange resins, purification with ethanol and charcoal, and direct crystallization of the fucose from ethanol.

Introduction

L-Fucose (L-galactomethylose, 6-deoxy-L-galactose) was first obtained in crystalline form by Günther & Tollens,[1] who isolated it from the products of hydrolysis of *Fucus* species through the intermediate phenylhydrazone. It was obtained in microscopic needles which had the composition $C_6H_{12}O_5$.

Widstoe & Tollens[2] isolated L-fucose from several samples of gum tragacanth; the white varieties on hydrolysis yielded fucose and arabinose, and brown tragacanth gave fucose and xylose. The existence of L-fucose in tragacanth was confirmed by James & Smith,[3] who isolated 2 : 3 : 4-trimethyl-α-methyl-L-fucoside from the hydrolysis products of methylated tragacanthic acid.

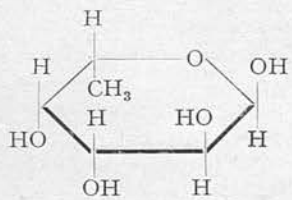
L-Fucose has been shown to be present in the specific blood-group substances. This was first demonstrated by Bray, Henry & Stacey[4] in their methylation studies on the group-A specific substance from commercial pepsin. They showed that the polysaccharide part of this glyco-polypeptide was composed of D-galactose, D-mannose, D-acetylglucosamine and L-fucose, the residues of acetylglucosamine and fucose being present as terminal groups in a highly branched structure. The existence of fucose in the blood-group substances has since been confirmed by a number of workers, such as Bendich, Kabat & Bezer[5] and Partridge.[6] Aminoff, Morgan & Watkins[7] have shown that the carbohydrate part, which makes up about 80% of the whole, of the human blood-group A substance from ovarian-cyst fluid contains one L-fucose, one D-galactose and two hexosamine (acetyl-D-glucosamine and acetyl-D-chondrosamine) residues, there being about 280 such units in each molecule. The polypeptide part, constituting about 20% of the whole, contained at least 11 amino-acids. Recently, Watkins & Morgan[8] have found that α-methyl-L-fucopyranoside, and to a lesser extent L-fucose, when mixed with the H-agglutinin in eel serum, neutralize completely the power of the antibody to agglutinate group O cells. This is the first instance of the inhibition by a simple sugar of a specific antigen–antibody reaction, where both components occur naturally.

Frog-spawn mucin also contains L-fucose.[9] The polysaccharide part (42% of the whole) of this mucin contains L-fucose, D-mannose, D-glucose, D-xylose, D-galactose, D glucosamine and D-chondrosamine, and the peptide portion contains some 18 amino-acids.

Vasseur,[10] and Vasseur & Immers,[11] showed that the jelly substance from certain sea-urchin eggs was composed of a polyfucose sulphate in combination with protein. The polysaccharide had high ash (over 40%) and sulphate (up to 32%) contents, and was considered to be esterified by one sulphate group per monosaccharide residue. Some idea of the structure of the polysaccharide has been obtained by oxidation with periodate.[12] This carbohydrate obviously shows some resemblance to fucoidin.

The presence of L-fucose has also been reported in the gum from *Acacia sieberiana*, a Belgian Congo tree,[13] and in the cell-wall of the flax fibre.[14]

L-Fucose crystallizes from water in fine microscopic needles (m.p. 145°), which mutarotate in aqueous solution from $[\alpha]_D^{20}$ — 152·6° (initial reading) to — 75·9°.[15, 16] The crystalline form is therefore α-L-fucose (I, α-L-fucopyranose).



(I)

Fucose is present in the brown seaweeds as the cell-wall constituent fucoidin, which is believed to be essentially a polyfucose monosulphate,[17, 18] e.g. $(C_6H_9O_3SO_4Ca_{0\cdot5})_n$. Hydrolysis of fucoidin with acid, followed by the removal of excess acid and inorganic salts, should yield an aqueous solution of fucose, from which the sugar should crystallize directly on evaporation. The isolation of fucose in this way has been investigated in this paper. The production of fucoidin from brown marine algae has been investigated by Black, Dewar & Woodward.[19]

Because of the early difficulties in obtaining quantities of fucoidin, previous workers[15, 20, 21] have used the seaweed itself as a source of fucose. The method employed is essentially that described originally by Tollens and co-workers,[1, 2] whereby the seaweed (*Ascophyllum nodosum*) is treated first with cold dilute hydrochloric acid, and then boiled with dilute sulphuric acid to effect hydrolysis. After separation of the weed residue, excess acid is removed either with barium or calcium carbonate, the filtrate evaporated to small volume, impurities precipitated by addition of methanol and ether, and the fucose isolated as the crystalline phenylhydrazone. The hydrazone is readily decomposed with benzaldehyde, and the free sugar crystallized from absolute ethanol. Bates and associates[15] fermented the filtrate, after neutralization with calcium carbonate, with baker's yeast acclimatized to ferment galactose, which was supposed to remove the mannose and galactose present in the solution. This treatment, however, would appear to be unnecessary, as galactose has not been identified in quantity in the products of hydrolysis of the Phaeophyceae, and only one reference appears in the literature to the presence of mannose in the brown seaweeds.[22]

Hockett, Phelps & Hudson[21] quote a yield of 39–60 g. of crystalline fucose from 1000 g. of anhydrous, hydrochloric acid-treated *A. nodosum*. On the assumption that the original untreated weed had a fucose content of 7%, and that the preliminary acid wash has removed 33% of the original weed,[23] this yield works out at 37–57% of the total fucose present. Clark[20] obtained 38–40 g. of crystalline fucose from 1000 g. of acid-treated *A. nodosum*, and Bates and associates[15] quote a yield of 30 g. from 1000 g. of water-washed *A. nodosum*.

Experimental; discussion of results

Preparation of L-*fucose from dried milled* Pelvetia canaliculata

The weed was collected at Atlantic Bridge, Seil Island, Argyllshire, in March, 1949, dried on a rack at 25–30° for 48 hours, and ground in a Christy & Norris mill fitted with a $\frac{1}{64}$-in. mesh screen. Fucose was estimated in the weed colorimetrically,[24] and the other constituents by the methods previously employed by one of the authors.[25] The analysis of the weed is shown in Table I.

The milled weed (1018 g.) was hydrolysed with 4% (w/v) sulphuric acid (8 l.) at 100° for three hours, the hydrolysate was worked up, and the crystalline fucose prepared as described

Table I

Percentage chemical composition (dry basis) of Pelvetia canaliculata

Fucose (as $C_6H_{12}O_5$)	Ash	Mannitol	Laminarin	Organic nitrogen
11·2	24·1	8·5	2·48	1·55

by Hockett, Phelps & Hudson.[21] The preliminary hydrochloric acid washing of the weed was omitted because cold acid treatment is known to extract appreciable quantities of fucoidin, e.g. when this weed was stirred with 10 volumes of 0·16N-hydrochloric acid at pH 1·5 in the cold for six hours, 19·8% of the total fucose present was extracted.[19] On the other hand, preliminary cold acid treatment removes the bulk of the mineral matter, which is therefore eliminated before hydrolysis and does not contaminate the fucose phenylhydrazone precipitate. Again, cold acid extracts most of the mannitol in the weed, thereby rendering the concentrated hydrolysate less viscous, and the hydrazone is more readily filtered from the mother liquor. It is recommended, therefore, in spite of the loss of fucose, that the weed be washed initially with cold hydrochloric acid to remove the major portion of the ash and other soluble material.

In the experiment, 126·7 g. of fucose phenylhydrazone was obtained, but the precipitate was badly contaminated with mineral matter (ash, 24·7%). After decomposition of the hydrazone with benzaldehyde, the fucose crystallizing out still had a high ash content (ash, 20·6%). Most of the mineral matter was eventually removed by dissolving the fucose in hot absolute ethanol, and filtering off the salts. The filtrate, on evaporation *in vacuo*, gave a syrup (42·5 g.) which was taken up in hot absolute ethanol (43 ml.), and the solution was allowed to crystallize in the refrigerator at 5° for two days. The yield of crystalline fucose A, after filtration, washing with ethanol and drying *in vacuo* over phosphorus pentoxide, was 31·5 g. (Found : ash, 1·9%). The yield, based on the total weight of fucose in the weed, is 28%, which is considerably less than that obtained by Hockett, Phelps & Hudson.[21]

The sugar A (30·8 g.) was recrystallized by dissolving in water to give an approximately 80% solution, diluted with absolute ethanol (2 ml. for each gram of sugar), and placed in the refrigerator to crystallize.[15] The fucose B was filtered off, washed with absolute ethanol, and dried. The mother liquor was evaporated to dryness, the syrup dissolved in absolute ethanol (2 ml. per gram of syrup), allowed to crystallize in the refrigerator and the fucose C isolated. The analysis of the two fractions is recorded in Table II.

Table II

Recrystallization of fucose A

Recrystallized fucose	Wt. as % of fucose A	Ash, %	M.p., ° c.	Equilibrium $[\alpha]_D^{17}$ in water (*c.* 4·1)
Fucose B	39·0	0·29	137–8	− 75·0°
Fucose C	36·2	0·18		− 74·6°

Hockett, Phelps & Hudson[21] quote a 70% recovery for the recrystallization of fucose from absolute ethanol.

Preparation of L-*fucose from fucoidin*

The three samples of fucoidin used in these investigations were prepared by the method of Black, Dewar & Woodward,[19] and gave the analysis shown in Table III.

Table III

Analysis of fucoidin samples

Sample	Species extracted from :	Fucose (as $C_6H_{12}O_5$), %	Ash, %	$[\alpha]_D^{20}$ in water (*c*, 0·992 ; 2-dm. tube)
Fucoidin A	*Fucus vesiculosus*	39·9	35·6	− 118°
Fucoidin B	*F. vesiculosus*	34·9	36·3	
Fucoidin C	*P. canaliculata*	41·1	32·0	

Rate of hydrolysis of fucoidin with acids

The rate of liberation of L-fucose from fucoidin A was followed by estimating the reducing power in the hydrolysate with Shaffer & Somogyi's method.[26] The reducing power of fucose solutions, when estimated with Shaffer & Somogyi reagent 50, is linearly related to the fucose content,[27] although L-fucose has a much lower reducing power than D-glucose (1 mg. of fucose = 4·37 ml. of 0·005N-$Na_2S_2O_3$; 1 mg. of glucose = 9·02 ml. of 0·005N–$Na_2S_2O_3$).

The results are recorded in Table IV. In Expt. 1 the fucoidin A was heated under reflux on a boiling-water bath with the hydrochloric acid, 2-ml. portions of the hydrolysate were withdrawn at intervals and allowed to cool. Solution (1 ml.) was then transferred by pipette to a weighed beaker, the weighed solution diluted with water (20–30 ml.), neutralized with

0·1N-sodium hydroxide (phenol red as indicator), and made up to 100 ml. The reducing power of 5-ml. portions of this solution was then estimated with Shaffer & Somogyi reagent 50 against a 5-ml. water blank as described by Cameron, Ross & Percival.[28]

$$\% \text{ Conversion to fucose} = \frac{(\text{Titration diff.}) \times 100 \times (\text{total wt. of soln.}) \times 100 \times 100}{4{\cdot}37 \times 5 \times (\text{wt. of 1 ml. of soln.}) \times 39{\cdot}9 \times 1000}$$

In Expt. 2, the hydrolysis was carried out under the same conditions, but the rate of liberation of fucose was followed by estimating the acetaldehyde liberated on treatment with periodate. Hydrolysate (1 ml.) was weighed, made up to 10 ml. in a standard flask, and 5 ml. of this solution was neutralized with solid sodium bicarbonate and the fucose determined by the standard procedure.[24] In this way, the results obtained by the reducing power method can be calibrated against the true fucose figures. The results obtained by the reducing power method are invariably too high in the final stages of hydrolysis, due to the presence of small quantities of galactose, xylose and uronic acid in fucoidin,[17] which exert a reducing action on the copper reagent but which do not liberate acetaldehyde on treatment with periodate.

In Expts. 3 and 4 the fucoidin was dissolved in hydrochloric acid in a Lintner pressure bottle fitted with a rubber stopper, and the hydrolysis was carried out at 135° in a Pentecon autoclave. The reducing power was estimated as before.

The results in Table IV show that the liberation of fucose from fucoidin with 0·57N-hydrochloric acid at 100° is complete after two hours, whereas with 0·25N-hydrochloric acid at 135° hydrolysis is complete after one hour.

Table IV

Rate of hydrolysis of fucoidin A with acids

Expt. No.	Wt. of fucoidin A hydrolysed, g.	Acid used	Concn. of fucoidin, % (w/w)	Temp. of hydrolysis, °c.	% conversion to fucose after time (hr.) 0·25	0·50	1·0	1·5	2·0	3·0	5·0	7·5
1	1·962	0·57N-HCl, 20·41 g.	8·77	100		62·2	81·0		106·3	118·8	125·0	134·1
2	1·962	0·57N-HCl, 20·41 g.	8·77	100		70·5	90·1		93·5	92·5	86·4	89·4
3	3·296	0·05N-HCl, 17·20 g.	16·07	135	4·3	8·5	14·3					
4	3·292	0·25N-HCl, 17·14 g.	16·12	135	41·1	61·6	112·7	134·6	132·9			

The concentration of acid in the solution increases during hydrolysis owing to the splitting of the ethereal sulphate grouping, and this was readily detected in neutralizing with 0·1N-sodium hydroxide before estimating reducing power.

$$\begin{bmatrix} C_6H_9O_3SO_4^- \\ C_6H_9O_3SO_4^- \end{bmatrix} Ca^{2+} \xrightarrow{\text{complete hydrolysis } (4H_2O)} 2C_6H_{12}O_5 + H_2SO_4 + CaSO_4$$

In a concentrated solution, such as Expt. 4, the normality of sulphuric acid liberated is considerable and may often exceed that of the original hydrolysis acid. The slow rate of hydrolysis in Expt. 3 is probably due to the presence of some sodium alginate in fucoidin A, which on treatment with hydrochloric acid will give insoluble alginic acid, and hydrogen ions will be removed from solution. The hydrolysis after one hour was found to be almost neutral, indicating that insufficient hydrochloric acid had been added. In all these experiments, an insoluble residue was formed during hydrolysate. Percival & Ross[17] found 3·3% of uronic acid in the hydrolysis products of their highly purified fucoidin from *Himanthalia lorea*.

Preparation of L-*fucose from fucoidin by the phenylhydrazone procedure*

Fucoidin C (10·07 g.) was hydrolysed with 0·5N-sulphuric acid (400 ml.) at 100° for four hours, neutralized with barium carbonate, filtered, the barium salts were thoroughly washed with boiling water, and the filtrate and washings evaporated *in vacuo* at 50°. The yellow glass was dissolved in water (40 ml.), ethanol (400 ml.) was added, the brown sticky precipitate was centrifuged off, and the centrifugate evaporated to a syrupy glass (5·15 g.). The glass was dissolved in water (7 ml.) and ethanol (45 ml.), phenylhydrazine (5·5 g., i.e. 2·0 mol.) and glacial acetic acid (1·0 ml.) were added, and the solution was maintained at 5° for two days. The crystalline fucose phenylhydrazone was filtered off, washed with ethanol (2 × 10 ml.),

and dried *in vacuo* over phosphorus pentoxide to a yellowish-white solid (3·202 g.). Yield, 49·9%; m.p. 170–172°.

The phenylhydrazone (3·175 g.) was decomposed by suspending in water (65 ml.), benzaldehyde (1·68 g., i.e. 1·3 mol.) was added, and the mixture was heated at 90° for one hour with frequent stirring. After cooling to 5°, the benzaldehyde phenylhydrazone was filtered off, the filtrate extracted with chloroform (3 × 10 ml.) to remove benzoic acid and excess benzaldehyde, and evaporated *in vacuo* to a pale-yellow syrup (2·17 g.). This was dissolved in absolute ethanol (3 ml.), when the fucose began to crystallize almost immediately. After three days at 5°, the crystals were filtered, washed with ethanol (2 × 1 ml.) and dried *in vacuo* over phosphorus pentoxide to a white powder (1·824 g.). Details of the product are shown in Table V.

Table V

Crystalline L-*fucose from hydrolysed fucoidin C*

Yield from phenylhydrazone, %	Yield from fucoidin C, %	Ash, %	M.p., ° c.	Equilibrium $[\alpha]_D^{17}$ in water (*c*, 2·04)	Purity (calc. from rotation), %
89·0	44·4	3·7	131–3	− 73·0°	96·1

The percentage purity was calculated assuming the $[\alpha]_D^{20}$ of L-fucose at equilibrium to be − 76·0° in water.[21] The product was still slightly contaminated with mineral matter, and this is reflected in the low melting-point. From the results it is apparent that the most serious loss of yield is at the formation of the phenylhydrazone, where 50% of the fucose in the hydrolysate is lost. In a similar experiment using methylphenylhydrazine (3·3 mol. per mol. of fucose present), fucose methylphenylhydrazone was formed in 90·4% yield, which was increased to 96·8% when corrected for a 93·4% yield of the methylphenylhydrazone from pure fucose.[24]

Preparation of L-*fucose from fucoidin by direct crystallization*

Since in the phenylhydrazone procedure a serious loss occurs at the formation of the hydrazone, an attempt has been made to crystallize fucose directly from the hydrolysate after removal of salts and acid with ion-exchange resins. Zeo-Karb 225 and Amberlite 1R-4B-OH were found to be satisfactory as cation- and anion-exchanger respectively. Amberlite 1RA-400-OH, however, was found to absorb fucose almost quantitatively, and the solution after treatment contained little or no sugar. This strongly basic resin must react with fucose in some way, perhaps to form a *N*-glycoside, e.g.:

$$\underset{\text{Fucose}}{CH_3\cdot[CH\cdot OH]_4\cdot CHO} + \underset{\text{resin}}{R\cdot NH_2} \longrightarrow \underset{N\text{-glycoside}}{CH_3\cdot[CH\cdot OH]_4\cdot CH{:}NR} + H_2O$$

In neutralizing fucoidin hydrolysates, care must be taken to add sufficient Amberlite 1R-4B-OH to deal not only with the acid used for hydrolysis but also with the sulphuric acid liberated from the ethereal sulphate group in fucoidin. A typical hydrolysis was carried out as follows:

Fucoidin C (5·030 g.) was hydrolysed with 0·5N-sulphuric acid (400 ml.) at 100° for four hours, and the solution centrifuged to remove a small brown precipitate. The colourless centrifugate was passed through a Zeo-Karb 225 column, the effluent neutralized by adding Amberlite 1R-4B-OH and stirring for about 30 minutes, and the resin filtered off and washed thoroughly with water. The filtrate and washings were evaporated *in vacuo* at 50° to a syrup, which was dissolved in water (20 ml.), ethanol (200 ml.) was added, and the light-brown precipitate removed at the centrifuge. Further impurities were removed by evaporating the centrifugate again to dryness, extracting the syrup with absolute ethanol (50 ml.), and centrifuging from the undissolved material. The alcohol was evaporated to give a yellow syrup which was dissolved in water (50 ml.), decolorized with charcoal (2 g.) at 100° for 30 minutes, filtered, and the filtrate evaporated *in vacuo* to dryness. The colourless syrup (2·32 g.) was dissolved in absolute ethanol (4 ml.) and the solution placed in the refrigerator, when crystals formed immediately. After two days at 5°, the crystalline fucose A was filtered, washed with ethanol (2 × 1 ml.), and dried to a white powder (1·170 g.).

The mother liquor was evaporated to a syrup, which was dissolved in water (25 ml.), decolorized with charcoal (1 g.) as before, and the resulting syrup (0·99 g.) crystallized from ethanol (1 ml.). After three days at 5°, crystalline fucose B was isolated (0·409 g.). The analysis of these two products is shown in Table VI.

The yield of fucose A (56·6%) is an improvement on the yield (44·4%) obtained by the phenylhydrazone procedure, and this method also gives considerable saving in time and

Table VI

Crystalline fucose fractions from hydrolysed fucoidin C

Fraction	Yield from fucoidin C, %	Ash, %	Equilibrium $[\alpha]_D^{17}$ in water (c, 2·02)	Purity (calc. from rotation), %
Fucose A	56·6	0·4	− 72·3°	95·1
Fucose B	19·8	0·2	− 63·1°	83·0

materials. The fucose B obtained from the mother liquor is rather impure, and would require further purification.

Recrystallization of L-*fucose*

Fucose can be readily purified by treatment with charcoal and recrystallizing from three volumes of ethanol.[21] L-Fucose (1·405 g.; equilibrium $[\alpha]_D^{17}$ in water, − 71·6°) was dissolved in water (25 ml.), the solution was heated with charcoal (1 g.) at 100° for 30 minutes, filtered, and the filtrate evaporated to dryness. The colourless syrup (1·327 g.) was dissolved in absolute ethanol (4 ml.), allowed to crystallize at 5° for three days, and the fucose isolated as a pure-white powder (1·139 g.). Yield, 81·1%; m.p., 135–137°; equilibrium $[\alpha]_D^{17}$ in water, − 76·1° (c, 2·05).

Large-scale preparation of L-*fucose from impure fucoidin*

In this large-scale experiment with impure fucoidin B, optimum conditions of hydrolysis were employed (Table IV; Expt. 4). Fucoidin B (91·7 g.) was dissolved in 0·25N-hydrochloric acid (478 ml.) to give a 16·1% (w/w) fucoidin solution, and the solution hydrolysed at 135° for 2 hours. The hydrolysate was centrifuged to remove a brown residue, cations were removed with Zeo-Karb 225 and the acids absorbed with Amberlite 1R-4B-OH, and impurities removed with alcohol treatment as described previously. The resulting brown syrup was dissolved in water (340 ml.), decolorized with charcoal (36 g.), and the filtrate evaporated *in vacuo* at 40° to dryness. The colourless syrup (33·3 g.), which was dried thoroughly *in vacuo* over phosphorus pentoxide, was dissolved in ethanol (30 ml.), seeded with crystalline L-fucose, and kept at 5° for four days with occasional stirring. The crystalline fucose A was filtered, washed with ethanol (3 × 20 ml.), and dried *in vacuo* over phosphorus pentoxide to a white solid.

The mother liquor on evaporation gave a glass (12·8 g.) which was dissolved in water (12 ml.), and the solution treated with ethanol (80 ml.), phenylhydrazine (17 ml.) and glacial acetic acid (2·5 ml.), and kept at 5° for one day. The fucose phenylhydrazone was isolated (6·50 g.), and this was decomposed with benzaldehyde as described previously to give crystalline fucose B as a white solid. The analysis of these two fractions is shown in Table VII.

Table VII

Crystalline fucose fractions from hydrolysed fucoidin B

Fraction	Dry wt., g.	Yield from fucoidin B, %	Ash, %	Equilibrium $[\alpha]_D^{17}$ in water	Purity (calc. from rotation), %
Fucose A	18·90	54·2	0·8	− 71·1° (c, 4·22)	93·6
Fucose B	2·85	8·2	± 0·0	− 73·4° (c, 2·36)	96·6

The total yield of crystalline fucose therefore amounts to 62·4% of that present in fucoidin B. With larger quantities, the fucose would be crystallized from concentrated aqueous solutions.[15]

Summary

Crystalline L-fucose has been prepared in 28% yield from dried milled *P. canaliculata* by the method described by Hockett, Phelps & Hudson.[21] It is recommended that the seaweed be given a preliminary wash with cold dilute hydrochloric acid before hydrolysis to remove the major proportion of the inorganic salts and other soluble material which otherwise contaminate the fucose phenylhydrazone precipitate and render its separation a difficult problem.

The preparation of L-fucose from fucoidin has also been investigated. The complete hydrolysis of fucoidin is effected by heating a 16% (w/w) solution of the polysaccharide in 0·25N-hydrochloric acid at 135° in an autoclave for two hours. When the course of the hydrolysis is followed by estimating reducing power, the results indicate 'over-hydrolysis' in the final stages owing to the presence of small quantities of sugars other than L-fucose in the fucoidin molecule. Results obtained by the reducing power method must therefore be 'calibrated' against the true fucose figures obtained by estimating the acetaldehyde liberated on treatment with periodate.

Fucoidin, on hydrolysis, neutralization with barium carbonate and treatment with phenylhydrazine, gave fucose phenylhydrazone (yield 49·9%), from which crystalline fucose was obtained in 44·4% yield and 96·1% purity.

Fucose has been crystallized directly from fucoidin hydrolysates without the preparation of the intermediate phenylhydrazone. After hydrolysis, salts and acids are removed by ion-exchange resins, further impurities are eliminated by ethanol precipitation and treatment with charcoal, and the fucose crystallized from absolute ethanol. Fucoidin from *P. canaliculata* gave crystalline fucose in 56·6% yield and 95·1% purity; the yield, calculated on the weight of original weed, is 35·8%. The mother liquor gave a second crop of crystals in 19·8% yield and 83·0% purity.

In a hydrolysis involving 92 g. of impure fucoidin (fucose, 34·9%) from *F. vesiculosus*, crystalline fucose has been obtained in 54·2% yield and 93·6% purity by direct crystallization. The mother liquor gave a further 8·2% yield (purity, 96·6%) by the phenylhydrazone procedure, making a total yield of 62·4%.

Pure L-fucose can be obtained from the products described above by treating with charcoal and recrystallizing from three volumes of ethanol.

Acknowledgment

The work described in this paper forms part of the programme of research and development on seaweed undertaken by the Institute of Seaweed Research, and the authors are indebted to the Institute for permission to publish.

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Received 24 October, 1952

References

[1] Günther, A. & Tollens, B., *Ber. dtsch. chem. Ges.*, 1890, **23**, 2585

[2] Widstoe, J. A. & Tollens, B., *Ber. dtsch. chem. Ges.*, 1900, **33**, 132

[3] James, S. P. & Smith, F., *J. chem. Soc.*, 1945, p. 739

[4] Bray, H. G., Henry, H. & Stacey, M., *Biochem. J.*, 1946, **40**, 124

[5] Bendich, A., Kabat, E. A. & Bezer, A. E., *J. Amer. chem. Soc.*, 1947, **69**, 2163

[6] Partridge, S. M., *Biochem. J.*, 1948, **42**, 251

[7] Aminoff, D., Morgan, W. T. J. & Watson, W. M., *Biochem. J.*, 1950, **46**, 426

[8] Watkins, W. M. & Morgan, W. T. J., *Nature, Lond.*, 1952, **169**, 825

[9] Folkes, B. F., Grant, R. A. & Jones, J. K. N., *J. chem. Soc.*, 1950, p. 2136

[10] Vasseur, E., *Acta chem. scand.*, 1948, **2**, 900

[11] Vasseur, E. & Immers, J., *Ark. Kemi*, 1949, **1**, 39

[12] Vasseur, E., *Acta chem. scand.*, 1952, **6**, 376

[13] Adriaens, L., *Mém. Inst. colon. belge Sci. nat.*, 8°, 1939, **8**, 1

[14] Cashmore, A. E., *J. chem. Soc.*, 1927, p. 718

[15] Bates, F. J. & associates, 'Polarimetry, Saccharimetry and the Sugars', Circular C440 of the National Bureau of Standards, 1942, p. 460 (Washington, D.C.: U.S. Govt. Printing Office)

[16] Pigman, W. W. & Goepp, R. M., 'Chemistry of the Carbohydrates', 1948, p. 107 (New York: Academic Press Inc.)

[17] Percival, E. G. V. & Ross, A. G., *J. chem. Soc.*, 1950, p. 717

[18] Conchie, J. & Percival, E. G. V., *J. chem. Soc.*, 1950, p. 827

[19] Black, W. A. P., Dewar, E. T. & Woodward, F. N., *J. Sci. Fd Agric.*, 1952, **3**, 122

[20] Clark, E. P., *J. biol. Chem.*, 1922, **54**, 65

[21] Hockett, R. C., Phelps, F. P. & Hudson, C. S., *J. Amer. chem. Soc.*, 1939, **61**, 1658

[22] Manske, R. H. F., *J. biol. Chem.*, 1930, **86**, 571

[23] Black, W. A. P., Cornhill, W. J., Dewar, E. T. & Woodward, F. N., *J. appl. Chem.*, 1951, **1**, 505

[24] Black, W. A. P., Cornhill, W. J., Dewar, E. T., Percival, E. G. V. & Ross, A. G., *J. Soc. chem. Ind., Lond.*, 1950, **69**, 317

[25] Black, W. A. P., *J. Soc. chem. Ind., Lond.*, 1948, **67**, 165

[26] Shaffer, P. A. & Somogyi, M., *J. biol. Chem.*, 1933, **100**, 695

[27] Cameron, M. C., Ross, A. G. & Percival, E. G. V., unpublished results

[28] Cameron, M. C., Ross, A. G. & Percival, E. G. V., *J. Soc. chem. Ind., Lond.*, 1948, **67**, 161

Reprinted from Chemistry and Industry, 1954, *pp.* 514–516

D-GALACTOSE

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D-Galactose occurs abundantly in nature as a component of pectins, gums and mucilages. It has been found in a great variety of plant material where it occurs chiefly as a galactan. Agar and carragheenin, for example, extracts of the red seaweeds (Rhodophyceae), have been shown to be galactan sulphates which give 30—50% of D-galactose on acid hydrolysis.

Yanagawa[1] prepared crude agar from six species of red seaweeds by extracting with hot water and precipitating the agar with alcohol. Galactose assays by the mucic acid method gave results from 37% in the agar from *Gelidium subcostatum* to 48% in that from *Gracilaria confervoides*, while commercial agar gave a value of 41%. From *Gelidium amansii* Araki[2] prepared crude agar containing 32% of galactose, while Hayashi[3] found 30% galactose in a more impure preparation from *G. amansii*.

The presence of DL-galactose in *Porphyra laciniata*, one of the most important seaweed foods of Japan, was reported by Oshima and Tollens[4] as early as 1901. Pirie[5] isolated a crystalline derivative of DL-galactose from commercial agar, which also contained 0·8% of L-galactose. DL-galactose has also been isolated from the hydrolysates of the polysaccharides obtained from *Bangia fusco-purpurea*, *Porphyra crispata* and *P. tenera* by hot water extraction.[6]

D-Galactose has recently been obtained from lactose, agar and the red seaweed *Gracilaria confervoides*.[7] From the acid hydrolysate of lactose or agar, after deacidification by electrolysis and evaporation to a syrup, galactose crystallized out in 31% and 28% yield respectively. From *G. confervoides*, by a somewhat similar procedure, galactose was obtained in 17% yield.

Recently it has been shown by Ross[8] that practically every species of red seaweed with any appreciable sulphate content contains galactose as the primary sugar, the exceptions being the genera Rhodymenia (dulse) and Rhodochorton which contain xylose, and Corollina which contains glucose as the main sugar.

With the likelihood of an increasing demand for antibiotics where lactose is used in the substrate, the possibility of using galactose as a substitute has been considered, while other outlets for this sugar have also been envisaged. Consequently, a number of red seaweeds, indigenous to Britain, have been analysed for galactose, while terrestrial plant material such as Sphagnum, peat, heather and bracken, has also been examined.

The method of analysis used, of which complete details will shortly be published, consists essentially in

Table I

% *Composition of miscellaneous samples (dry basis)*

Sample	Total reducing substances calc. as anhydro hexoses	% Galactose	% Glucose	% Xylose	% Arabinose
Gigartina stellata					
North Berwick, 10.6.52	41·3	21·1	N.D.	N.D.	*
Furcellaria fastigiata					
Galway Bay, 15.12.51	29·2	18·5	5·1	3·7	*
Porphyra umbilicalis					
North Berwick, 24.4.52	43·2	16·6	N.D.	N.D.	*
Ceramium rubrum					
North Berwick, 10.6.52	30·3	14·7	10·5	3·3	*
Polysiphonia fastigiata					
North Berwick, 10.6.52	N.D.	9·6	N.D.	N.D.	*
Cast weed					
Worthing, 17.8.53	23·9	9·3	8·2	4·2	*
Sphagnum					
Dunmore, 3.7.53	28·4	11·0	5·9	5·6	2·9
Heather (whole plant) (in bud)					
Culross, 15.7.53	28·1	6·1	N.D.	5·4	1·7
Bracken fronds and stipes					
Culross, 15.7.53	25·2	3·7	11·3	1·0	2·4
Bracken rhizomes					
Culross, 15.7.53	32·3	4·7	17·3	1·6	3·9
Hop bine					
(Whitbreads)	24·3	3·6	N.D.	14·4	N.D.
Dunmore peat					
Stirlingshire, 3.7.53	42·3	10·5	8·3	3·9	5·0
Benhar peat					
Lanarkshire	11·8	4·9	4·4	5·9	N.D.
Bargeddie peat					
Lanarkshire	16·1	4·3	6·5	4·3	N.D.
Gardrum peat					
Stirlingshire	17·9	4·1	4·3	3·8	N.D.
Halladale peat					
Sutherland	14·9	4·6	2·6	N.D.	N.D.

BROWN ALGAE. N.D

N.D.—not determined. *—absent.

running, with controls, a one-dimensional chromatogram for 48 hours (Tate and Lyle solvent[9]) of an acid. hydrolysate of the sample, cutting the strips out, eluting with cold water,[10] and determining the individual sugars with hypoiodite.[11] By this method a synthetic mixture of glucose and galactose was separated and recoveries of 98% and 95% achieved for glucose and galactose respectively. The results obtained are given in Table I.

From these figures it can be seen that the best sources of galactose are the red seaweeds used in the production of agar and carragheenin, although *Sphagnum* and the young Dunmore peat contain reasonable amounts. The older, more decomposed peats, however, contain considerably less galactose. The bracken rhizomes are exceptional in that, after hydrolysis, they contain 17% of glucose.

With the exception of the wartime survey of the littoral zone on certain British shores for *Chondrus crispus* and *Gigartina stellata*,[12] no quantitative assessment of the world's littoral and sublittoral red seaweed resources appears to have been made.

There is no doubt however that these are appreciable as, at the present time, at least 14,000 tons of dry agar- and carragheenin-containing red marine algae are collected and processed annually in thirteen maritime countries.[13]

Little is known about Britain's underwater red seaweed resources although tens of thousands of tons are cast up each summer on the South Coast in the Worthing area where it is causing great concern, as it decomposes rapidly and is responsible for plagues of flies. A sample of this cast weed, collected in August, 1953, was found to contain 9·3% galactose after hydrolysis.

This work forms part of the programme of research and development on seaweed undertaken by the Institute of Seaweed Research, to whom the authors are indebted for permission to publish.

Received March 18, 1954

References

[1] Yanagawa, T., *Chem. Abstr.*, 1936, **30,** 3541

[2] Araki, C., *J. chem. Soc. Japan*, 1937, **58,** 1214

[3] Hayashi, K., *Chem. Abstr.*, 1948, **42,** 5425

[4] Oshima, K. & Tollens, B., *Ber. dtsch. chem. Ges.*, 1901, **34,** 1422

[5] Pirie, N. W., *Biochem. J.* 1936, **30,** 369

[6] Hayashi, K., Nogaku (Sci. of Agr.), 1948, **2,** 226

[7] Mizuguchi, J., Suzuki, S. & Misono, S., *J. agric. chem. Soc., Japan*, 1952, **26,** 461

[8] Ross, A. G., *J. Sci. Fd. Agric.*, 1953, **7,** 333

[9] Albon, N. & Gross, D., *Analyst*, 1950, **75,** 454

[10] Laidlaw, R. A. & Reid, S. G., *Nature, Lond.*, 1950, **166,** 476

[11] Hirst, E. L., Hough, L. & Jones, J. K. N., *J. chem. Soc.*, 1949, 928

[12] Marshall, S. M., Newton, L. & Orr, A. P., " A Study of certain British Seaweeds and their utilisation in the preparation of agar," 1949 (London: Her Majesty's Stationery Office)

[13] Woodward, F. N. (in the press)

(4617) PRINTED BY HARRISON AND SONS, LTD., ST. MARTIN'S LANE, LONDON

THE PROPERTIES OF THE ALGAL CHEMICALS. I.—The Evaluation of the Common British Brown Marine Algae as a Source of Alginate

By W. A. P. BLACK, W. J. CORNHILL and E. T. DEWAR

An investigation has been carried out to evaluate the common brown algae indigenous to Great Britain as a source of alginate. In particular, the viscosity of the sodium alginate from each species has been examined, and the effect on the viscosity of drying and bleaching has been studied. In an attempt to show that the alginates from the different species are essentially the same, specific rotations have been determined.

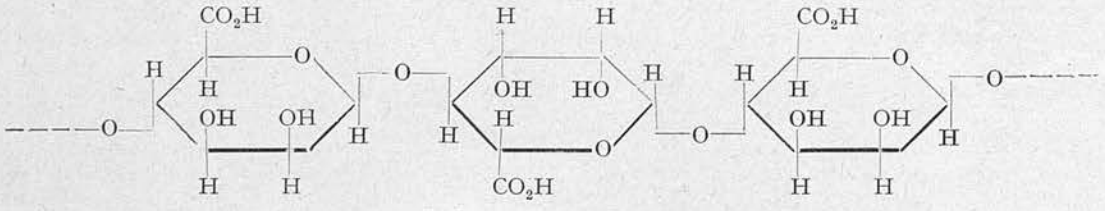
Introduction

Alginic acid was discovered by Stanford (1883), who gave the name ' algin ' to the viscous solution obtained by treating Laminariaceae with dilute sodium carbonate. The gelatinous precipitate obtained on filtering and acidifying the viscous extract was termed ' alginic acid ' (Stanford, 1884*a*). The acid was found to give a series of well-defined salts (Stanford, 1884*b*, 1886; Kylin, 1915; Hoagland, 1915; Gómez, 1933; Novodranov, 1935).

Stanford believed alginic acid to be a nitrogenous compound, but later work by Krefting (1896), Oden (1917), Nelson & Cretcher (1929), Miwa (1930) and Gómez (1933) has shown it to be nitrogen-free.

Hoagland & Lieb (1915) subjected alginic acid to acid hydrolysis, and isolated from the hydrolysate a pentosazone which they believed to be L-xylosazone. Atsuki & Tomoda (1926) suggested that the acidic nucleus in alginic acid was glucuronic acid, the cinchonine salt of which Schmidt & Vocke (1926) claimed to have isolated from the hydrolysis products. Nelson & Cretcher (1928, 1929), however, showed that alginic acid was a polyuronide in which all the aldehyde groups were conjugated and all the carboxyl groups free. They doubted the presence of xylose, and believed that any pentose found on hydrolysis could have arisen from the decarboxylation of the uronic acid unit. On hydrolysis the uronic acid was identified as D-mannuronic acid, and later both D-mannuronic acid lactone and the corresponding D-mannosaccharic acid dilactone were isolated in crystalline condition (Nelson & Cretcher, 1930). Bird & Haas (1931) and Miwa (1930) independently identified mannuronic acid as the main constituent unit in alginic acid. By improved methods of preparation, Schoeffel & Link (1933) isolated the free α- and β-D-mannuronic acids in crystalline condition, and their identification was established beyond doubt by comparison with synthetic D-mannuronic acid lactone obtained by reduction of D-mannosaccharic acid dilactone (Niemann & Link, 1933). Using 90% formic acid for hydrolysis, Spoehr (1947) prepared mannuronic acid lactone in 50% yield.

Although it is extremely difficult to obtain ether-type or ester-type derivatives of alginic acid, Hirst, Jones & Jones (1939) succeeded in methylating completely a partially degraded alginic acid. Hydrolysis with methanolic hydrogen chloride yielded the methyl ester of 2 : 3-dimethyl D-mannuronoside, thereby indicating that alginic acid is a straight-chain polymannuronide containing β-D-mannopyruronic acid residues linked through C_1 and C_4. The pyranose structure (I) was favoured in view of the extreme stability of alginic acid and its large negative rotation.



(I)

Similar conclusions on the structure of alginic acid have been obtained by Lucas & Stewart (1940) using periodic acid, Astbury (1945) using *X*-ray analysis, and recently by Chanda, Hirst, Percival & Ross (1952). Numerous investigators have determined the neutralization equivalent of alginic acid, and their results are discussed by Rose (1937). He obtained a value of 194 for the free acid, giving an empirical formula of $(C_6H_8O_6H_2O)_n$, whereas the theoretical

equivalent weight of a pure polymannuronide, $(C_6H_8O_6)_n$, with all carboxyl groups free, is 176. The values (179) found by Nelson & Cretcher (1929) and Miwa (1930) were the only ones approaching this theoretical figure. Cameron, Ross & Percival (1948) and Rose (1951), however, have used 176 in their recent work, and this has been adopted throughout this paper. This gives the equivalent weight of sodium alginate as 198.

The optical rotation of alginic acid has been determined by a number of investigators, and the results are summarized in Table I. Some of the original results have been recalculated on the basis of sodium alginate, using 176 as the equivalent of the free acid.

Table I

Specific rotation of sodium alginate

Reference	Species	$[\alpha]_D$ in water
Hoagland & Lieb (1915)	*Macrocystis pyrifera*	− 150°
Kylin (1915)	*Laminaria digitata*	− 122°
	Ascophyllum nodosum	− 120°, − 121°
Atsuki & Tomoda (1926)	*L. saccharina*	− 116°
Schmidt & Vocke (1926)	*Fucus serratus*	− 131°
Nelson & Cretcher (1929)	*M. pyrifera*	− 133°
Colin & Ricard (1930)	*L. flexicaulis*	− 119°
Miwa (1930)	*Undaria pinnatifida*	− 133°
Schoeffel & Link (1933)	*F. serratus*	− 121°
Gómez (1933)	*L. flexicaulis, F. vesiculosus, A. nodosum*	− 130·5°, − 132°
Barry & Dillon (1936)	*L. digitata*	− 144·6°
Rose (1937)	Commercial sodium alginate	− 135·3°
Hirst *et al.* (1939)	Commercial sodium alginate	− 139°

The specific rotation of sodium alginate is high, and the more recent values obtained by different workers from various sources are in fair agreement, varying from − 130° to − 145°. Rose (1937) found the specific rotation of sodium alginate was independent of grade, and therefore independent of molecular weight, over a fairly wide range. He obtained a mean value of − 135·3°, but when the alginate was extensively degraded by hydrolysis with 0·5N-hydrochloric acid at 100° for 5 hours, the rotation fell to − 128° and the sample showed reducing properties.

By viscometric measurements Heen (1937, 1938) calculated the molecular weight of sodium alginate to be 15,000; Donnan & Rose (1950), using osmotic pressure and viscosity measurements, obtained molecular weights varying from 48,000 to 186,000 for different grades of sodium alginate.

The most outstanding property of alginic acid is the high viscosity shown by aqueous solutions of its soluble salts even at quite low concentrations (0·25–1·0%), and it is chiefly this property which has made the alginates so important in recent years in the food, pharmaceutical, medical, dental, textile, rubber, paint and paper industries. The various uses of the alginates in industry have been discussed by Tseng (1946), McDowell (1949) and Tressler & Lemon (1951).

The 'grade' of commercial sodium alginate is generally defined by the viscosity of a 1% solution at 25°. Commercial alginate samples on the market vary widely in grade, from 50 to 1500 centistokes (cs.), different products being manufactured to suit various applications in industry.

The viscosity of sodium alginate solutions has been investigated by Diáz & Aenlle (1950), who found that the viscosity decreased with time of standing. They attributed this to spontaneous depolymerization of the sodium alginate, and the effect was minimized by the addition of 0·1–0·2% phenol in the solution.

The first attempt to evaluate the various brown seaweeds as a source of alginate was made by Rose (1951). He rated the following Canadian seaweeds in order of decreasing suitability as sources of alginate: *Laminaria digitata, Nereocystis luetkeana, Macrocystis integrifolia, Ascophyllum nodosum* and *Fucus vesiculosus*. The yield and quality of alginate were found to be very sensitive to the method and conditions of extraction and purification. From a commercial standpoint the value of any sample of seaweed as a source of alginate depends upon its alginate content, the cost of extraction, and the quality of the product. Quality depends upon colour, purity and viscosity.

The production of alginates from brown marine algae has been investigated by Bashford, Thomas & Woodward (1950), and the variation of the alginic acid content of the common species of Laminariaceae and Fucaceae with season of the year and depth of immersion has been studied by Black (1948*a*, *b*, 1949, 1950*a*, *b*).

Experimental; discussion of results

The methods employed are essentially those of Rose (1937, 1951), although a few modifications have been introduced.

Recommended procedure for the extraction of alginate and determination of the viscosity of 0·25% sodium alginate solution

Dried milled ($\frac{1}{64}$-in. screen) or fresh minced ($\frac{3}{16}$-in. plate) weed (dry weight, 1 g.) was treated at 60° for 30 minutes with water (20 ml.) containing calcium hydroxide (0·05 g.), centrifuged and washed with water (2 × 10 ml.). The residue was treated in the cold with 0·2N-sulphuric acid (20 ml.) for 30 minutes, and then centrifuged and washed with water (10 ml.). The residue was digested at 50° with 3% sodium carbonate (10 ml.) for 2 hours with occasional stirring, diluted with water (90 ml.), stirred rapidly for 3 hours in the cold and left overnight. The solution was then centrifuged, and the residue washed with water (2 × 10 ml.).

The viscous centrifugate and washings were treated (when bleaching was required) with N-sodium hypochlorite (1·2 ml.) in the cold for 30 minutes, and the alginate precipitated by running the solution slowly from a separating funnel, with stirring, into 25% (w/v) calcium chloride (10 ml.). The calcium alginate was filtered (1 × 1 crucible), washed with dilute sulphur dioxide solution (1 vol. of saturated sulphur dioxide solution diluted to 10 vol.), extracted with 0·5N-hydrochloric acid (3 × 100 ml.) until free from calcium ions during 30 minutes only, and the alginic acid washed with water (3 × 100 ml.) until free from chloride ions.

The alginic acid was then rinsed into a weighed 250-ml. beaker and titrated with 0·1N-sodium hydroxide using phenolphthalein as indicator. From the titration figure obtained, the percentage of alginic acid ($C_6H_8O_6$) can be obtained.

A factor of 100/96 is introduced to compensate for loss due to the 1 × 1 crucible (Cameron, Ross & Percival, 1948).

The weight of sodium alginate in solution = volume of 0·1N-sodium hydroxide × 0·1 × 0·1981 g. The titrated solution was then weighed and diluted with water to give an 0·25% (w/w) sodium alginate solution, sodium chloride was added to bring the normality to 0·1, and the viscosity measured at 25° in Ostwald viscometers (see B.S. 188 : 1937).

The method is essentially that worked out by Rose (1951). Digestion with 3% sodium carbonate was carried out at 50° instead of 20°, as low yields were obtained at 20° when the results were compared with those obtained with the standard analytical procedure (Cameron, Ross & Percival, 1948). This is revealed in Table II.

Table II

Comparison of the yields obtained by the methods of Rose (1951) *and Cameron, Ross & Percival* (1948)

Species	Alginic acid (dry basis), %		
	Method of Rose (1951)		Method of Cameron, Ross & Percival (1948)
	20° digestion	50° digestion	
F. vesiculosus (FV/XII/45/ME)	12·1	13·7	15·0
A. nodosum (A/VI/45/O)	19·5*	22·9 20·6*	24·4
L. digitata frond (D/X/45/L frond)	19·9 19·0*		19·3
L. cloustoni stipe (C/III/49 stipe)	18·3*	23·1	21·9

* Results after bleaching with sodium hypochlorite.

The low results obtained with the Fucaceae after bleaching are discussed elsewhere. Digestion at 50° removed 93·8% of the alginic acid from *A. nodosum*, and this is generally considered one of the most difficult species to extract. Digestion at 50° was therefore considered satisfactory for a general procedure.

When bleaching was required, a constant quantity (1·2 ml.) of N-sodium hypochlorite was used for each gram-sample of weed. This was found to be sufficient to give a white alginic acid for all the species examined (Rose, 1951, Table VI, method 4).

Rose titrated his alginic acid samples with stirring in a Waring Blendor. It was found that rapid stirring was not essential, however, although with slower stirring a slightly longer time was required for the titration. End-points were normally sharp, except in the case of unbleached *A. nodosum* where the brown colour of the solution tended to make the end-point difficult to observe.

The sodium chloride was added to eliminate the electroviscous effect shown by sodium alginate solutions (Rose, 1937, 1951; Donnan & Rose, 1950). Rose found that 0·1N-sodium chloride swamps this effect, and higher concentrations, up to 0·5N-sodium chloride, either have no additional effect or slightly increased the viscosity of 0·1 to 0·5% solutions of sodium alginate.

Variation of viscosity of sodium alginate with rate of shear

Hydrophilic colloids do not, in general, obey Poiseuille's law, for their viscosity depends on the shearing force to which the solution is subjected. In general, the viscosity of such solutions decreases as the rate of shear is increased, because the long polymer molecules tend to orientate themselves with their long axes more nearly parallel to the streamlines of flow, thereby offering less resistance to flow (Frith & Tuckett, 1951). Consequently, results obtained with different types of instruments, or with the same instrument but different shearing forces, are not directly comparable.

Rose (1937) found that increasing the rate of shear had little effect on the viscosities of solutions of the lower sodium alginate polymers [12 to 80 centipoises (cp.) at 25° at 1% concentration], but it decreased the viscosities of the higher polymers (330 cp. at 1% concentration), e.g. an 0·5% solution of the latter high-grade sodium alginate had a viscosity of 40·4 cp. with a No. 2 B.S. viscometer at 25° and 37·6 cp. with a No. 3 viscometer. This effect was found to be more pronounced at higher concentrations of alginate, but Rose considered that the B.S. viscometers were suitable for viscosities below 40 cp. In the present investigation, the effect of rate of shear on viscosity was found to be much more marked, owing to the fact that the sodium alginate solutions examined had, in general, a much higher grade than those employed by Rose. The results are recorded in Table III.

Table III

The viscosity of 0·25% sodium alginate solutions in 0·1N-sodium chloride at 25° as measured with different viscometers

Expt. No.	B.S. viscometer No.	Viscometer constant, cs./sec.	Viscosity, cs.
1 ..	2	0·0575	15·3
	3	0·306	14·0
2 ..	2	0·0531	18·7
	3	0·306	16·7
3 ..	2	0·0531	20·3
	3	0·306	18·1
4 ..	2	0·0575	36·6
	3	0·306	30·8
5 ..	2	0·0531	41·3
	3	0·306	33·1
	2	0·0531	47·7
	2	0·0575	46·7
6 ..	3	0·306	37·4
	3	0·337	37·9
7 ..	2	0·0575	55·0
	3	0·306	43·7
8 ..	3	0·306	196·3
(0·50% sodium alginate solution)	4	1·451	172·0

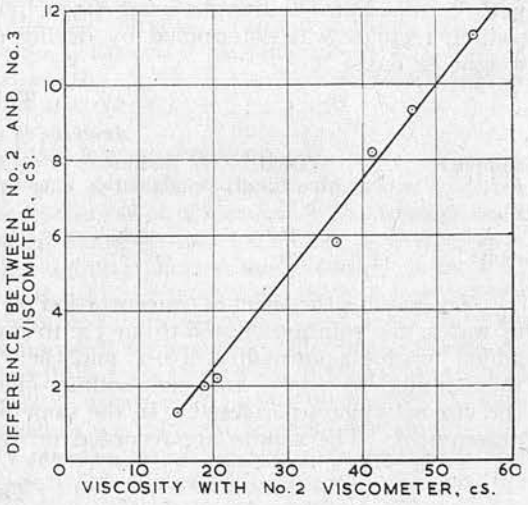


FIG. 1

The results show that the effect of shear increases with grade, and becomes significant above 20 cs. When the difference in viscosity between Nos. 2 and 3 viscometers is plotted against the viscosity with the No. 2 viscometer (Fig. 1), a rough linear relationship is obtained, from which the difference at any viscosity can be approximately deduced. At 20 cs. (No. 2 viscometer), the variation is about 2·5 cs.; at 30 cs., about 5 cs.; at 40 cs., about 7·5 cs.; and at 50 cs., about 10 cs.

Slight variations were also detected (Expt. 6) with viscometers of the same number but slightly different constants, although in general these were not significant.

The method finally adopted to compare the viscosities of the various alginates from different species was to use a No. 3 viscometer down to 20 cs., and a No. 2 viscometer between 5 and 20 cs. Where a No. 2 viscometer was used above 20 cs., the viscosity figures are corrected to a No. 3.

Effect of concentration on the viscosity of sodium alginate

The viscosity of sodium alginate solutions increases very rapidly with concentration, and viscosity–concentration curves for alginates of different grade have been determined by Donnan & Rose (1950). They found that these curves are all similar in shape and can be made to coincide throughout their length by selecting a suitable concentration scale for each one. The factors by which the concentration scales have to be multiplied to make curves coincide were referred to as 'relative concentration factors'. From a single reference curve, therefore, it is possible to construct the complete viscosity–concentration curve of any alginate sample by determining the viscosity of a single solution of known concentration.

In this investigation the effect of concentration on viscosity has been examined for two grades of sodium alginate at four concentrations. A high-grade alginate A was prepared from fresh *L. digitata* frond, which was collected at South Queensferry, Firth of Forth, on 1 October, 1951. After being minced three times in a Reliance meat mincer fitted with a $\frac{3}{16}$-in. plate, the frond (603 g.; dry weight, 75·1 g.; alginic acid, 22·7%) was extracted and the calcium alginate prepared exactly as described in the recommended procedure. The calcium alginate was leached with 0·5N-hydrochloric acid (3 × 1 l.), washed with water (3 × 2 l.), and the alginic acid titrated with 2N-sodium hydroxide (47 ml.) using phenolphthalein as indicator. The viscous sodium alginate (2700 ml.) was added slowly with rapid stirring to ethanol [4050 ml.; i.e. 60% (v/v) concentration], the stringy precipitate filtered on muslin, washed with ethanol and ether, and dried *in vacuo* over phosphorus pentoxide (14·6 g.). A lower-grade sodium alginate B was prepared from the same *L. digitata* sample, which had been first dried at 60–80° for 4½ hours and then milled through a $\frac{1}{64}$-in. screen. The analysis of A and B is given in Table IV. The sodium alginate content was determined by dissolving about 0·2 g. in 0·3% sodium carbonate (100 ml.), and adding the solution to 25% calcium chloride (10 ml.). The calcium alginate was then leached with 0·5N-hydrochloric acid, washed with water, and titrated with 0·1N-sodium hydroxide using phenolphthalein as indicator. The moisture content of sodium alginate was determined by drying *in vacuo* over phosphorus pentoxide to constant weight (2 days).

Table IV

Analysis of samples A and B

Sample	Yield from frond, %	Sodium alginate, %	Ash (as sulphate), %	Calc. ash (as Na_2SO_4) for $C_6H_7O_6Na$, %	Viscosity of 0·25% solution in 0·1N-NaCl at 25°, cs.
A..	76	97·0	34·9	35·87	30·8
B..	71	98·8	34·7	35·87	22·4

To examine the effect of concentration on viscosity, sodium alginate (0·503 g.) was dissolved in water, the solution diluted to 50·3 g. to give a 1% (w/w) solution, sodium chloride (0·294 g.) added to give a normality of 0·1, and the viscosity measured at 25°. The 1% solution was then diluted to 0·75%, sufficient sodium chloride added to maintain a normality of 0·1, and the viscosity again measured. In the same way, the viscosities at 0·50 and 0·25% (w/w) were determined. The results are recorded in Table V, and the curves are plotted in Fig. 2.

Table V

Viscosity of sodium alginate solutions A and B at various concentrations

Concn., %	0·25		0·50		0·75		1·00	
	A	B	A	B	A	B	A	B
Viscometer No.	3	3	4	3	4	4	4	4
Viscometer constant, cs./sec.	0·306	0·306	1·451	0·306	1·451	1·451	1·451	1·451
Viscosity, cs.	30·8	22·4	282·1	196·3	1501	830·1	4837	2593

Although it is difficult to describe these curves accurately with only four points, it can be seen that A and B are similar in shape. When the horizontal scale for sample A is multiplied by 1·15, curve A roughly coincides with curve B.

Comparison of the viscosities of sodium alginate from different species

The viscosity of an 0·25% sodium alginate solution has been determined for each species using (1) fresh minced weed; (2) dried milled weed without bleaching; and (3) dried milled weed with bleaching of the sodium alginate.

The Fucaceae were collected at South Queensferry on 18 September, 1951. Each species was minced twice in a Reliance meat mincer fitted with a $\frac{3}{16}$-in. plate, and samples weighed out

for dry weight and viscosity determination. The viscosity was determined exactly as described in the recommended procedure, except that bleaching with sodium hypochlorite and washing with sulphur dioxide were omitted. The remainder of the minced species was dried for 7 hours at 60–80°. The dried weed was then milled through a $\frac{1}{64}$-in. screen and used for Expts. (2) and (3).

The Laminariaceae were collected off Inchcolm in the Firth of Forth on 5 September, 1951. Each species was separated into frond and stipe, and minced separately. Samples were dried at 60–80° for $4\frac{1}{2}$ hours only.

The results are summarized in Table VI.

An important point revealed in these experiments is the marked loss of alginic acid content with bleaching of the Fucaceae. This is not apparent to any extent with the Laminariaceae, but with *A. nodosum* the drop in alginic acid on bleaching amounts to over 16%. Even with hydrogen peroxide, which is a much less drastic bleaching agent, the result was the same. An unsuccessful attempt was made to bleach the coloured solution from *A. nodosum* with ultra-violet light. This effect has been observed by Rose (1951), who suggests that the loss of titratable material indicates that unbleached alginate contains an acidic impurity which is easily oxidized by hypochlorite. This is of some commercial significance in the case of *A. nodosum*, because the alginic acid content (from the point of view of obtaining alginic acid free from colour) must be taken as 20·0 and not 23·9%.

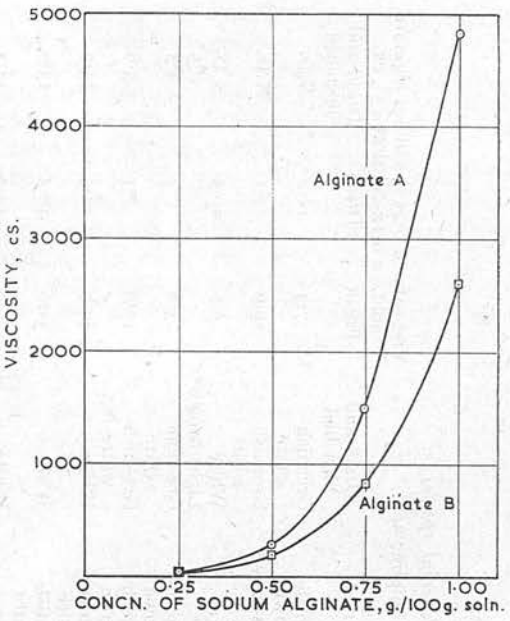


FIG. 2

Comparison of the specific rotations of sodium alginate from different species

The specific rotation of each sodium alginate was determined on the bleached solution, as it was impossible to see through the unbleached solution at a suitable concentration, with the exception of *L. digitata*. The dried milled weed samples were extracted as described in the recommended procedure, and the crude sodium alginate solution was bleached in the cold with N-sodium hypochlorite (1·2 ml.) for 30 minutes. The calcium alginate was precipitated as before, washed with dilute sulphur dioxide solution, calcium ions were removed with 0·5N-hydrochloric acid and hydrochloric acid was removed with water. The white gelatinous alginic acid was titrated in a weighed beaker with 0·1N-sodium hydroxide, sufficient water was added to bring the concentration of sodium alginate to about 0·5%, and the weighed solution thoroughly shaken in a stoppered flask. The solution was allowed to stand overnight, and the rotation measured in a 1-dm. tube. With *P. canaliculata, F. spiralis, F. vesiculosus* and *F. serratus*, the solutions had to be filtered through a dry sintered-glass crucible (G.3) before reading the rotation to remove a slight turbidity from the greenish solutions. The results are recorded in Table VII. The specific rotations are accurate to $\pm 2°$.

All the results lie within the range $-139 \pm 10°$, which is in good agreement with the more recent values of $-144{\cdot}6°$ (Barry & Dillon, 1936), $-135{\cdot}3°$ (Rose, 1937) and $-139°$ (Hirst *et al.*, 1939).

Effect of bleaching on the specific rotation of sodium alginate

To study the effect of bleaching on the rotation of sodium alginate, dried milled *L. digitata* frond (D/X/45/L; 2·009 g.) was extracted as before and the crude alginate divided into two portions. Aliquot A was bleached with sodium hypochlorite; B was unbleached. The rotations and viscosities of both solutions were determined and recorded in Table VIII.

Rose (1937) found the specific rotation of sodium alginate was independent of grade over the range of viscosity 4–160 cp. for an 0·5% solution at 25°, and this has been confirmed for these two samples.

Effect of different conditions of drying on the viscosity of sodium alginate from L. digitata *frond*

Because of the very marked drop in viscosity with the Laminariaceae after drying for

Table VI

Comparison of the viscosities of sodium alginate from different species

	Alginic acid (dry basis), %			Colour of alginic acid			Viscosity of 0·25% sodium alginate soln. in 0·1N-NaCl at 25°, cs.		
	Fresh	Dried	Dried and bleached	Fresh	Dried	Dried and bleached	Fresh	Dried	Dried and bleached
Pelvetia canaliculata	16·3	19·7	16·9	Greenish-brown	Greenish-brown	Greenish-white	7·3	11·0	N.D.
F. spiralis	12·0	14·4	13·1	Light-brown	Light-brown	Greenish-white	10·9	12·1	N.D.
A. nodosum	23·4	23·9	20·0 19·8*	Dark-brown	Brown	White Light-brown*	6·8	10·5	N.D. 8·5*
F. vesiculosus	N.D.	14·5	13·2	Greenish-brown	Greenish-brown	Greenish-white	5·8	7·1	4·3
F. serratus	N.D.	19·2	17·0	Greenish-brown	Greenish-brown	Greenish-white	7·6	7·4	5·6
L. cloustoni frond	14·1	14·1	13·5	Light-brown	Greenish-white	White	44·9	18·5	15·9
L. saccharina frond	18·9	19·6	19·3	Greenish-white	Greenish-white	White	31·6	18·5	13·0
L. digitata frond	N.D.	20·8	20·5	White	White	White	72·0	15·0	11·5
L. cloustoni stipe	N.D.	20·5	20·3	Light-brown	Light-brown	White	16·9	10·0	6·2
L. saccharina stipe	19·5	23·5	22·9	Light-brown	Light-brown	White	19·2	12·1	7·6
L. digitata stipe	28·5	30·8	30·6	Greenish-white	Greenish-white	White	57·9	14·4	11·1

N.D. = Not determined.
* Bleaching carried out with 30% (w/v) hydrogen peroxide (1·0 ml.) in the cold for 1½ hours in place of sodium hypochlorite.

Table VII

The specific rotations of sodium alginate from different species

Species	$[\alpha]_D$	Concn. of sodium alginate, g./100 g. of soln.
P. canaliculata	− 138	0·470
F. spiralis	− 142	0·558
A. nodosum	− 129	0·435
F. vesiculosus	− 142	0·521
F. serratus	− 148	0·505
L. cloustoni frond	− 133	0·474
L. saccharina frond	− 135	0·614
L. digitata frond	− 133	0·463
L. cloustoni stipe	− 141	0·496
L. saccharina stipe	− 134	0·456
L. digitata stipe	− 143	0·498

Table VIII

The specific rotation of bleached and unbleached sodium alginate

Aliquot	Concn. of sodium alginate, g./100 g. soln.	$[\alpha]_D$	Viscosity of 0·25% sodium alginate solution in 0·1N-NaCl at 25°, cs.
A	0·543	− 138° (± 2°)	9·4
B	0·494	− 134° (± 2°)	25·9

4½ hours at 60–80° (Table VI), it was decided to investigate further the effects of drying for one particular species. The *L. digitata* frond was collected at South Queensferry on 1 October, 1951. A sample was minced through a $\frac{3}{16}$-in. plate, and the viscosity determined on the fresh frond.

A sample of fresh frond was dried *in vacuo* over anhydrous calcium chloride for 3 days to 11·5% moisture, and milled through a $\frac{1}{64}$-in. screen. A portion of this material was further dried *in vacuo* over phosphorus pentoxide for 2 days to 1·5% moisture.

A sample of fresh frond was air-dried by hanging it at an open window for 5 days to 22·4% moisture, and chopped into small pieces. Portions of this material were dried for 4½ hours at 27°, 40° and 60–80°, and then milled through a $\frac{1}{64}$-in. screen. A portion of the 60–80° sample was further heated at 100° for 17 hours. To study the effect of heat on pure sodium alginate, sample A (Table IV ; viscosity of 0·25% solution in 0·1N-sodium chloride at 25°, 30·8 cs.) was heated at 40° for 4½ hours and at 100° for 17 hours. The results obtained on the various samples are recorded in Table IX.

Table IX

Effect of different drying conditions on the viscosity of sodium alginate

Expt. No.	Drying conditions	Moisture, %	Alginic acid (dry basis), %	Viscosity of 0·25% soln. in 0·1N-NaCl at 25°, cs.
1	Fresh frond	87·5	23·2	42·8
2	Vacuum-dried over $CaCl_2$	11·5	23·6	41·2
3	Vacuum-dried over P_2O_5	1·5	24·1	33·1
4	Air-dried	22·4	24·6	43·7
5	Dried at 27°	15·9	25·5	15·3
6	Dried at 40°	13·8	24·9	18·7
7	Dried at 60–80°	7·9	24·5	18·1
8	Dried at 100°	—	23·6	16·0
9	Soln. from (2) reprecipitated in 25% $CaCl_2$, leached and redissolved	—	—	37·4
10	Soln. from (4) heated at 40° for 4½ hr.	—	—	44·3
11	Soln. from (10) allowed to stand in stoppered flask for 13 days	—	—	43·1
12	Sodium alginate A heated at 40°	—	—	24·0
13	Sodium alginate A heated at 100°	—	—	4·8

It would appear that any form of thermal drying leads to depolymerization of the high-grade alginate present in the fresh weed (Expts. 5–8). Air-drying (Expt. 4) or drying in a vacuum desiccator over calcium chloride (Expt. 2), however, gives no loss of grade, and drying over phosphorus pentoxide to almost anhydrous conditions gives only a slightly lower viscosity (Expt. 3). The viscosities of 15–19 cs. obtained for these heated samples are in fair agreement with the values (10–18·5 cs.) obtained for the thermally dried, unbleached Laminariaceae in Table VI. Heating pure sodium alginate in the dry state also leads to a loss of grade (Expts. 12 and 13), 17 hours at 100° causing a very large drop in viscosity.

The effect of reprecipitating a high-grade alginate was studied in Expt. 9. The sodium alginate from Expt. 2 was reprecipitated in 25% calcium chloride, the calcium alginate extracted with 0·5N-hydrochloric acid, and the alginic acid washed with water, titrated with 0·1N-sodium hydroxide, and the viscosity determined. The viscosity dropped only from 41·2 to 37·4 cs., thereby indicating that leaching of the calcium alginate with hydrochloric acid does not seriously lower the grade.

Although heating the weed and the dry sodium alginate at 40° for 4½ hours causes degradation (Expts. 6 and 12), heating the pure sodium alginate solution under the same conditions (Expt. 10) does not affect the grade. A high-grade alginate solution showed very little loss of viscosity after 13 days at room temperature (Expt. 11) ; in another experiment (not recorded in Table IX), an 0·25% solution having a viscosity of 22·4 cs. at 25° in 0·1N-sodium chloride, gave a viscosity of 21·2 cs. after 24 days' standing and there was no sign of mould growth in the solution.

Summary

The viscosities of an 0·25% sodium alginate solution in 0·1N-sodium chloride at 25° have been determined for *P. canaliculata, F. spiralis, A. nodosum, F. vesiculosus, F. serratus, L. cloustoni, L. saccharina* and *L. digitata*. With the fresh Laminariaceae, viscosities vary between 17 and 72 cs., with the frond for each species higher than the corresponding stipe. It is significant that *L. cloustoni* stipe gives the poorest grade of all the Laminariaceae. With the fresh Fucaceae, no great difference in grade is apparent, results varying between 5·8 and 10·9 cs. Drying the Laminariaceae at 60–80° before extraction produces a marked loss of

grade for the high-viscosity samples, all the results for the dried species lying between 10 and 18·5 cs. With the Fucaceae, however, drying at 60–80° gives a slight increase in grade, viscosities varying between 7·1 and 12·1 cs. The effect of bleaching on the sodium alginate solution is to produce a further drop in viscosity for all species examined, but the alginic acid obtained is almost free from colour. Bleaching with sodium hypochlorite, or with hydrogen peroxide, leads to an apparent loss of alginate with the Fucaceae.

Although no work has yet been carried out, it is quite possible that the viscosity of sodium alginate extracted from the fresh species will show a variation with season of the year, depth of immersion, habitat, stage of development of the plant, and also along the length of the plant.

The specific rotations of the sodium alginate from each species have been determined, and all the figures lie within the range $-139 \pm 10°$, which is in good agreement with the more recent values quoted in the literature.

The effect of different conditions of drying on the grade of the resulting alginate has been studied for *L. digitata* frond. Air-drying the frond, or drying in a vacuum desiccator over anhydrous calcium chloride or phosphorus pentoxide, leads to no appreciable loss of grade, but thermal drying, even at 27° for 4½ hours, produces an alginate of much lower viscosity. On the other hand, a pure, high-grade sodium alginate solution is stable to heating at 40° for 4½ hours, and shows very little loss of viscosity after standing at room temperature for 13 days.

The viscosity of sodium alginate increases very rapidly with concentration and this effect has been investigated for two samples of different grade.

Acknowledgment

This work forms part of the programme of research and development of the Institute of Seaweed Research, and the writers are indebted to the Director for permission to publish.

The Institute of Seaweed Research
Inveresk
Midlothian

Received 9 April, 1952

References

Astbury, W. T. (1945). *Nature, Lond.* **155,** 667.
Atsuki, K. & Tomoda, Y. (1926). *J. Soc. chem. Ind., Japan* **29,** 509.
Barry, V. C. & Dillon, T. (1936). *Sci. Proc. roy. Dublin Soc.* **21,** 285.
Bashford, L. A., Thomas, R. S. & Woodward, F. N. (1950). *J. Soc. chem. Ind.* **69,** 337.
Bird, G. M. & Haas, P. (1931). *Biochem. J.* **25,** 403.
Black, W. A. P. (1948*a*). *J. Soc. chem. Ind.* **67,** 165, 169, 172.
Black, W. A. P. (1948*b*). *J. Soc. chem. Ind.* **67,** 355.
Black, W. A. P. (1949). *J. Soc. chem. Ind.* **68,** 183.
Black, W. A. P. (1950*a*). *J. Soc. chem. Ind.* **69,** 161.
Black, W. A. P. (1950*b*). *J. Mar. biol. Ass. U.K.* **29,** 45.
Cameron, M. C., Ross, A. G. & Percival, E. G. V. (1948). *J. Soc. chem. Ind.* **67,** 161.
Chanda, S. K., Hirst, E. L., Percival, E. G. V. & Ross, A. G. (1952). *J. chem. Soc.* p. 1833.
Colin, H. & Ricard, P. (1930). *Bull. Soc. Chim. biol., Paris* **12,** 1392.
Diáz, G. & Aenlle, E. O. (1950). *An. Soc. esp. Fís. Quím.* [B] **46,** 441.
Donnan, F. G. & Rose, R. C. (1950). *Canad. J. Res.* [B] **28,** 105.
Frith, E. M. & Tuckett, R. F. (1951). 'Linear Polymers', p. 220 (London: Longmans, Green & Co.).
Gómez, M. L. (1933). *Inst. esp. Oceanograf. Notas y resumenes* Ser. II, No. 74.
Heen, E. (1937). *Tidsskr. Kemi Bergv.* **17,** 127.
Heen, E. (1938). *Kolloidzschr.* **83,** 204.
Hirst, E. L., Jones, J. K. N. & Jones, W. O. (1939). *J. chem. Soc.* p. 1880.
Hoagland, D. R. (1915). *J. agric. Res.* **4,** 39.
Hoagland, D. R. & Lieb, L. L. (1915). *J. biol. Chem.* **23,** 287.
Krefting, A. (1896). Eng. Pat. 11,538.
Kylin, H. (1915). *Hoppe-Seyl. Z.* **94,** 337.
Lucas, H. J. & Stewart, W. T. (1940). *J. Amer. chem. Soc.* **62,** 1792.
McDowell, R. H. (1949). *Trans. Plast. Inst., Lond.* **17,** 62.
Miwa, T. (1930). *J. chem. Soc., Japan* **51,** 738.
Nelson, W. L. & Cretcher, L. H. (1928). *Science* new series, **67,** 537.
Nelson, W. L. & Cretcher, L. H. (1929). *J. Amer. chem. Soc.* **51,** 1914.
Nelson, W. L. & Cretcher, L. H. (1930). *J. Amer. chem. Soc.* **52,** 2130.
Niemann, C. & Link, K. P. (1933). *J. biol. Chem.* **100,** 407.
Novodranov, J. K. (1935). *Ann. Leningr. Univ.* Chem. Ser. **1,** 58.
Oden, S. (1917). *Int. Z. phys.-chem. biol.* **3,** 83.
Rose, R. C. (1937). Ph.D. Thesis, London University.
Rose, R. C. (1951). *Canad. J. Technol.* **29,** 19.
Schmidt, E. & Vocke, F. (1926). *Ber. dtsch. chem. Ges.* **59,** 1585.
Schoeffel, E. & Link, K. P. (1933). *J. biol. Chem.* **100,** 397.
Spoehr, H. A. (1947). *Arch. Biochem.* **14,** 153.
Stanford, E. C. C. (1883). *Chem. News* **47,** 254, 267.
Stanford, E. C. C. (1884*a*). *J. R. Soc. Arts* **32,** 717.
Stanford, E. C. C. (1884*b*). *J. Soc. chem. Ind.* **3,** 297.
Stanford, E. C. C. (1886). *J. Soc. chem. Ind.* **5,** 218.
Tressler, D. K. & Lemon, J. M. (1951). 'Marine Products of Commerce', 2nd ed., p. 94 (New York: Reinhold Publishing Corpn.).
Tseng, C. K. (1946). 'Colloid Chemistry' (Ed. J. Alexander), vol. 6, p. 629 (New York: Reinhold Publishing Corpn.).

Printed in Great Britain by Butler & Tanner Ltd., Frome and London

Reprinted from the Journal of The Science of Food and Agriculture, 1954, No. 4, pp. 176–183

THE PROPERTIES OF THE ALGAL CHEMICALS. II.*—Some Derivatives of Laminarin

By W. A. P. BLACK and E. T. DEWAR

The solubilization of the cold-water-insoluble form of laminarin from *Laminaria cloustoni* frond has been investigated. The hydroxyethyl, hydroxylpropyl and carboxymethyl ethers of laminarin have been prepared in good yield, and solutions of these in water show no sign of precipitation after being set aside for several weeks. The benzyl ether is water-insoluble. Laminarin triacetate, tribenzoate and tricarbanilate have also been prepared; these esters are insoluble in water but soluble in a wide range of organic solvents. A series of laminarin sulphates, containing from 2·15 to 0·20 sulphate groups per glucose unit, have been prepared, and the more highly sulphated esters are water-soluble.

Introduction

Laminarin prepared from *Laminaria cloustoni* frond[1, 2] is insoluble in cold water, and this property has been found to have many disadvantages when attempting to find uses for this polysaccharide. The present investigation, therefore, has been directed chiefly at the problem of solubilizing laminarin, although several water-insoluble derivatives have also been prepared. A few compounds of laminarin have been prepared by previous workers in their investigations into the structure of laminarin, e.g. the triacetate and trimethyl ether have been prepared by Barry,[3] and Connell *et al.*[1] prepared a monobenzoate in addition. Because of the similarity in chemical composition between laminarin and starch, most of the methods used in this paper are modifications of those that have been applied successfully for preparing the derivatives of starch, and these have been reviewed by Degering.[4]

Experimental and discussion of results

The laminarin used in these investigations was the insoluble form extracted from *L. cloustoni* frond;[2] it contained 0·7% of ash and gave $[\alpha]_D^{16}$ — 12·7° in water (*c*, 2·134). All rotations were measured in a 2-dm. tube.

Effect of sodium hydroxide on laminarin

Laminarin (1·49 g.) was dissolved in water (19 ml.) at 70°, cooled, 2N-sodium hydroxide (15 ml.) was added, and the solution was set aside at room temperature for 6 hours; the solution slowly turned yellow but no precipitate was formed. Hydrochloric acid (2N) was then added to give pH 4 and the solution was stirred, when the typical white precipitate of insoluble laminarin was slowly deposited. Sodium hydroxide was, therefore, ineffective as a means of solubilizing laminarin. Recently, some doubt has been thrown on the stability of laminarin towards alkali by Corbett *et al.*,[5] who have shown that insoluble laminarin is degraded by lime-water to a monobasic acid and a residue that is resistant to attack by lime-water. We have found that laminarin is attacked by 0·1N-sodium hydroxide, and these results will be discussed later.

Oxidation of laminarin

(1) *With hypoiodite.*—The method of Blair & Reeves[6] for converting the terminal aldehydic glucose units in hydrocellulose into gluconic acid was employed. Laminarin (3·065 g.) was

* Part I: *J Sci. Fd Agric.*, 1952, **3**, 542

dissolved in water (30 ml.) at 70°, the cold solution was buffered to pH 10·6 by adding sodium carbonate (0·716 g.) and sodium bicarbonate (0·036 g.), and treated immediately with 0·25N-iodine (15 ml.) containing 40 g. of potassium iodide per litre. A precipitate began to form within an hour. After 24 hours at room temperature, the solution was acidified with 2N-hydrochloric acid, set aside overnight, and the oxidized laminarin A was centrifuged, washed with water (10 ml.), ethanol (3 × 70 ml.) and ether (70 ml.), and dried *in vacuo* over phosphorus pentoxide to a white powder (2·632 g.). Analytical results are given in Table I. Oxidation of the reducing groups in laminarin, therefore, did not induce solubilization.

(2) *With hypochlorite.*—An amount equivalent to 0·5 atom of available oxygen per glucose unit was employed initially. Laminarin (2·719 g.; 16·78 mg. mol. of $C_6H_{10}O_5$) was dissolved in water (25 ml.) at 70°, the solution was cooled, 0·1N-sodium hydroxide (25 ml.) and 0·33N-sodium hypochlorite (50 ml., i.e. 8·25 mg. atoms of available oxygen) were added, and the solution was allowed to stand at room temperature in a stoppered flask for 2¼ hours, with occasional shaking. The specific rotation showed little change: $[\alpha]_D^{17}$ — 2·0° (initial) → — 2·6° (after 2 hours). The solution (pH 10·8) was then acidified with 2N-hydrochloric acid and dialysed for 2 days, when a precipitate was slowly deposited. The oxidized laminarin B was finally centrifuged and isolated, as above, as a white powder (1·203 g.). Analytical results are shown in Table I.

Table I

Analysis of oxidized laminarin

Fraction	Yield from laminarin, %	Ash, %	$[\alpha]_D^{16}$ in water (*c*, 2·0)
A	85·9	0·4	— 13·4°
B	44·2	0·4	— 13·3°

Craik[7] found that starch, on oxidation with hypochlorous acid for several days, was largely broken down into alcohol-soluble products which were strongly reducing.

In a second experiment, more drastic conditions were used in an attempt to effect solubilization. A quantity equivalent to 1·0 atom of available oxygen per glucose unit was employed, and the solution was heated at 40–45°. The reaction was followed polarimetrically by withdrawing 25 ml. of the solution at intervals, centrifuging to remove a small precipitate, and measuring the rotation. Solubility was then tested by acidifying the rotation solutions with 2N-hydrochloric acid, adding sufficient sodium sulphite ($Na_2SO_3,7H_2O$) to react with the chlorine liberated, and allowing the solutions to stand. The results are recorded in Table II.

Table II

Oxidation of laminarin with sodium hypochlorite at 40–45°

Time of oxidation, h.	$[\alpha]_D^{17}$ (*c*, 2·724)	Solubility on standing at room temperature
0·0	— 1·3°	—
1·0	— 4·2°	Copious white precipitate overnight
2·25	— 7·9°	Copious white precipitate overnight
4·0	— 16·3°	Copious white precipitate overnight
6·5	— 17·3°	Precipitation started after 2 days

These results show that laminarin is not readily solubilized by hypochlorite, and, as with starch,[7] the polysaccharide is probably undergoing considerable degradation.

Reduction of laminarin

Abdel-Akher *et al.*[8] have shown that insoluble laminarin from *L. cloustoni* can be transformed into a non-reducing polysaccharide, 'laminaritol', by reduction with 'sodium borohydride', a reagent that converts the aldehydic reducing group in sugars into the corresponding alcohols in aqueous solution. The following experiment, however, shows that reduction of laminarin did not induce solubilization.

Laminarin (0·502 g.) was dissolved in water (25 ml.) at 70°, the solution was cooled, sodium borohydride (0·177 g.) added, and the solution set aside at room temperature for 47 hours. The alkaline solution was then acidified with 2N-acetic acid, and, on being set aside, a precipitate began to form. After 3 days, the insoluble laminaritol was centrifuged and isolated as a dull white powder. Yield, 84·5%; $[\alpha]_D^{13}$ — 7·6° in water (*c*, 2·03).

These oxidation and reduction experiments indicate that laminarin cannot be solubilized by simply modifying the terminal reducing group in the chain, and results recorded below show that a major chemical reaction involving more or less every glucose unit is necessary to render the polysaccharide soluble.

Ethers of laminarin

Dr. A. G. Ross (unpublished work) has shown that laminarin can be solubilized by partial methylation with 40% sodium hydroxide and dimethyl sulphate, and the methyl ethers with a methoxyl content of more than 6% were soluble in water at a concentration of 6% to give solutions that were stable over a period of many weeks. In view of the rather excessive quantities of alkali required in this process, and of the need for dialysis to remove the large amount of sodium acetate after neutralization, the possibility of preparing other ethers of laminarin has been investigated.

(1) *Hydroxyethyl-laminarin.*—Ziese[9] has shown that starch readily reacts with ethylene oxide in aqueous alkaline solution at room temperature to give the monoglycol ether:

$$\underset{\text{Starch}}{C_6H_{10}O_5} + \underset{\substack{\text{Ethylene}\\\text{oxide}}}{(CH_2)_2O} \rightarrow \underset{\text{Monohydroxyethylstarch}}{C_6H_9O_5{\cdot}CH_2{\cdot}CH_2{\cdot}OH}$$

Lamarin was found to react similarly: Laminarin (2·997 g.) was dissolved in water (38 ml.) at 70°, cooled, 2N-sodium hydroxide (30 ml.) and ethylene oxide (3·0 ml.) were added, and the solution was kept at room temperature in a stoppered flask for 6 hours with occasional shaking; after 2 hours, a further 1·5 ml. of ethylene oxide was added. The yellow solution was neutralized by passing it through a Zeo-Karb 225 column, the effluent was adjusted to pH 7·6 with 0·1N-sodium hydroxide, and evaporated *in vacuo* at 50° to 36 ml. Acetone was then added to 85% (v/v) concentration, and the hydroxyethyl-laminarin was centrifuged, washed with ethanol and ether, and dried *in vacuo* over phosphorus pentoxide to a white powder (3·367 g.). Yield, 88·3% (assuming a monohydroxyethyl ether); $[\alpha]_D^{17}$ — 11·2° in water (*c*, 2·018) [Found: ash, 0·4%; C, 45·1; H, 7·31%. These results from the elementary analysis did not correspond too well with a monoglycol ether (calc. for $C_6H_9O_5{\cdot}CH_2{\cdot}CH_2{\cdot}OH$: C, 46·6; H, 6·84%), but this may be due to the presence of a small amount of water that has not been removed on drying *in vacuo* over phosphorus pentoxide].

This product dissolved readily in cold water to give a clear solution, and a 6% solution gave no precipitate when set aside for 26 days. A quantity of 148 g. of this derivative has been prepared, and is now under test as a possible substitute for blood plasma. In the large-scale preparation, the bulk of the sodium hydroxide was neutralized with Amberlite resin IRC-50-H, which is a weak acid resin with a high capacity, and the last traces were removed with Zeo-Karb 225. The alkali can also be eliminated, after neutralization, by dialysis, but the yield is less by this method.

(2) *Hydroxypropyl-laminarin.*—Laminarin has also been successfully solubilized by conversion into the hydroxypropyl ether:

$$\underset{\text{Laminarin}}{C_6H_{10}O_5} + \underset{\substack{\text{Propylene}\\\text{oxide}}}{CH_2{-}CH{\cdot}CH_3\ (\text{O})} \rightarrow \underset{\substack{\text{Mono-}(\beta\text{-hydroxy-}\\\text{propyl)laminarin}}}{C_6H_9O_5{\cdot}CH_2{\cdot}CH(CH_3){\cdot}OH}$$

When laminarin (3·002 g.) was treated with sodium hydroxide and propylene oxide exactly as described under (1), hydroxypropyl-laminarin was isolated as a white powder (3·094 g.). Yield, 75·9% (assuming a monohydroxypropyl ether); $[\alpha]_D^{15}$ — 11·8° in water (*c*, 2·04). Found: ash, 0·4%. Again the results of elementary analysis did not correspond exactly with those for a monoether. The product dissolved in cold water to give a clear solution, and a 6% solution gave no precipitate after being set aside for 36 days. A quantity of 66 g. of this material has been prepared in a larger-scale experiment, and this derivative is now under test as a possible substitute for blood plasma.

One advantage of these hydroxyalkyl ethers is that the number of hydroxyl groups per glucose unit remains unaltered, so that these derivatives can presumably still form trimethyl ethers and triacetates in the same way as the original laminarin.

(3) *Sodium carboxymethyl-laminarin.*—Chowdhury[10] has shown that various polysaccharides, including starch, react with chloroacetic acid in strongly alkaline solution to give the carboxymethyl (or glycollate) ether:

$$\underset{\text{Starch}}{C_6H_{10}O_5} + \underset{\substack{\text{Sodium}\\ \text{monochloroacetate}}}{ClCH_2{\cdot}CO_2Na} \rightarrow \underset{\substack{\text{Sodium}\\ \text{carboxymethylstarch}}}{C_6H_9O_5{\cdot}CH_2{\cdot}CO_2Na} + HCl$$

The conditions employed to give the monocarboxymethyl ether of starch were used for laminarin. The polysaccharide (2·973 g.) was dissolved in water (12 ml.) at 70°, cooled, 50% (w/w) sodium hydroxide (37 g.) was added slowly with shaking and cooling, followed by monochloroacetic acid (18·0 g.) added slowly with cooling, and the curdy, white mass was thoroughly shaken in a stoppered flask. After 19 hours at room temperature, the mixture was diluted to 200 ml., neutralized with glacial acetic acid, dialysed against running water for 4 days, and the solution was evaporated *in vacuo* at 50° to 45 ml. Saturated sodium chloride solution (0·5 ml.) was then added, followed by ethanol to 85% (v/v) concentration, and the sodium carboxymethyl-laminarin was centrifuged, washed with ethanol and ether, and dried *in vacuo* over phosphorus pentoxide to a dazzling white powder (4·084 g.). Found: ash (as Na_2SO_4), 27·6%, i.e. Na, 8·94%. This represents 0·91 sodium carboxymethyl grouping per glucose unit, and gives the formula weight of the repeating unit, $C_6H_9O_5(CH_2{\cdot}CO_2Na)_{0\cdot91}$, as 235. Yield, 94·7%; $[\alpha]_D^{20} - 7{\cdot}0°$ in water (*c*, 2·004).

The material was readily soluble in cold water, giving a stable solution, and was insoluble in organic solvents. A 2% aqueous solution formed insoluble salts with copper sulphate, silver nitrate, lead acetate and aluminium sulphate solutions, but gave no precipitate with calcium, barium, magnesium or manganous chloride solutions.

Dr. A. G. Ross (unpublished work) has found, by using different amounts of chloroacetic acid, that products with a much lower carboxymethyl content can be prepared, and the material containing 0·22 sodium carboxymethyl grouping per glucose unit gives a 6% aqueous solution which is stable for at least 21 days. Less-substituted products are unstable and are precipitated from aqueous solution within a few days. Sodium carboxymethyl-laminarin containing 0·22 side chain per glucose unit has been tested as a substitute for blood plasma.

Unsuccessful attempts have been made to carboxymethylate laminarin with less alkali, as in the preparation of the hydroxyethyl and hydroxypropyl ethers. Thus when laminarin (2·974 g., i.e. 18·34 mg. mol. of $C_6H_{10}O_5$, in 10 ml. of water), 2N-sodium hydroxide (30 ml., 60 mg. mol.) and monochloroacetic acid (2·848 g., 30·14 mg. mol.) were allowed to stand at room temperature for 19 hours, and the solution was neutralized and dialysed, a white precipitate began to be deposited in the dialysis bag within a few hours. After 2 days, the precipitate was centrifuged and isolated in 70·7% yield.

(4) *Benzyl-laminarin.*—The benzyl ethers of various carbohydrates, including starch, have been prepared by Gomberg & Buchler,[11] although complete benzylation was not readily achieved in the presence of only a slight excess of benzyl chloride. The object of preparing benzyl-laminarin was not an attempt to effect solubilization, for a benzyl ether would be expected to be water-insoluble, but simply to characterize the derivative. More drastic conditions than those employed by Gomberg & Buchler[11] were used.

Laminarin (2·502 g.; 15·44 mg. mol. of $C_6H_{10}O_5$) was dissolved in 20% (w/w) sodium hydroxide (18·5 g.; 93 mg. mol.), benzyl chloride (11·7 g.; 92 mg. mol.) was added, and the mixture heated under reflux at 80–85° for 4 hours with occasional shaking. After 17 hours at room temperature with mechanical shaking, the excess of benzyl chloride was removed by steam distillation (20 minutes), the solution was diluted to 80 ml., centrifuged, and the gummy product washed thoroughly with hot water containing a small quantity of sodium chloride. During the washing process the gum gradually solidified, and the resulting solid was dried *in vacuo* over phosphorus pentoxide (1·295 g.). This was purified by solution in glacial acetic acid (100 ml.) and precipitation with ether (200 ml.), and the benzyl-laminarin was centrifuged, washed with ether until free from acetic acid, and dried at 105° for one hour to a white powder (1·146 g.). $[\alpha]_D^{16} - 16{\cdot}0°$ in pyridine (*c*, 2·0) (Found: C, 55·7; H, 6·80%. Calc. for 0·5 benzyl group per glucose unit, $C_6H_{9\cdot5}O_5(CH_2{\cdot}C_6H_5)_{0\cdot5}$: C, 55·1; H, 6·33%).

The properties were very similar to those of benzylstarch.[11] Benzyl-laminarin was soluble in glacial acetic acid, pyridine and ethylene chlorohydrin, and insoluble in water, ether, ethanol, acetone, tetrahydrofuran, ethyl acetate, chloroform, benzene and light petroleum.

Esters of laminarin

(1) *Laminarin acetate.*—Acetyl-laminarin was first prepared by Barry,[3] who used both the sodium acetate/acetic anhydride procedure and the acetic acid/acetic anhydride/chlorine/sulphur dioxide method of Barnett. Connell *et al.*[1] dispersed the laminarin, which had been freshly precipitated by ethanol, in pyridine and acetylated with acetic anhydride in the cold, and this

method has been adopted in the experiments described below. Percival & Ross[12] have also used this method for acetylating soluble laminarin from *Laminaria digitata*.

As pointed out by Barry,[3] it is essential that the laminarin is in a finely divided state before acetylation by dissolving in water and precipitating with ethanol. Laminarin that has not been freshly precipitated gives a poor yield, as shown in the following experiment. Anhydrous laminarin (5·15 g.) was heated at 60° for 10 minutes with pyridine (52 ml.), cooled, and acetic anhydride (16 ml.) was added slowly with shaking; the polysaccharide swelled up but did not dissolve, and no heat was evolved. After 48 hours at room temperature the mixture was poured into water (500 ml.), and the acetate A was centrifuged, washed thoroughly with water (5 × 100 ml.), and dried *in vacuo* over phosphorus pentoxide to a white powder (3·19 g.). Yield (assuming a triacetate), 34·8%, $[\alpha]_D^9$ — 71·7° in chloroform (*c*, 1·018) (Found: Ac, 48·8%. Calc. for laminarin triacetate: $C_6H_7O_5Ac_3$: Ac, 44·8%). Acetyl content was determined by treating the acetate (0·1 g.) with A.R. methanol (10 ml.) and 0·1N-sodium hydroxide (25 ml.) at 60° for 20 minutes, in order to dissolve the acetate completely. After being set aside overnight in a stoppered flask, the solution was back-titrated with 0·1N-hydrochloric acid and phenolphthalein; a control was carried out simultaneously. Since the acetyl value was appreciably higher than the theoretical for the triacetyl derivative, a blank was run with laminarin itself; when 86·7 mg. was treated with methanol and sodium hydroxide exactly as described in the method, the consumption of alkali corresponded to 14·7% Ac or to an equivalent weight of 292. This result supports the findings of Corbett *et al.*,[5] who have found that laminarin is degraded by lime-water with the formation of a monobasic acid.

A good yield of triacetyl-laminarin was obtained in the following manner: Laminarin (2·434 g.) was dissolved in water (15 ml.), ethanol (200 ml.) was added, and the precipitate was centrifuged and washed thoroughly with ethanol and ether. The solid, still wet with ether, was heated with pyridine (33 ml.) at 60° for 10 minutes, cooled, and treated with acetic anhydride (10 ml.), when the solid dissolved completely to give an almost colourless solution. After being set aside for 48 hours at room temperature, when a certain amount of precipitation occurred, the mixture was poured into water, and acetate B was isolated as before, as a white powder (3·821 g.). Yield (assuming a triacetate), 88·3%. $[\alpha]_D^{17}$ — 63·6° in chloroform (*c*, 1·014) (Found: C, 49·3; H, 5·79; Ac, 47·4%. Calc. for $C_6H_7O_5Ac_3$: C, 50·0; H, 5·60; Ac, 44·8%).

Barry[3] obtained — 52°, and Connell *et al.*[1] — 60°, for the specific rotation of the triacetate in chloroform. Percival & Ross[12] found — 62° and — 65° for the acetates from insoluble and soluble laminarin respectively. Triacetyl-laminarin can be purified by solution in acetone and precipitation with light petroleum, but this treatment is not normally required. The product was soluble in chloroform, acetone, pyridine, ethyl acetate and glacial acetic acid; less soluble in benzene and tetrahydrofuran; and insoluble in water, ether, ethanol, carbon tetrachloride and light petroleum. The melting-point range was 190–220°.

A product with a lower acetyl content was obtained when the laminarin was dispersed in pyridine by the method employed by Pacsu & Mullen[13] for starch. The polysaccharide (5·01 g.) was dissolved in water (25 ml.), pyridine (150 ml.) was added, and the pyridine–water azeotrope distilled off at 50°/15 mm. until the volume of distillate was 125 ml., to eliminate the water; on being cooled, the solution gave a white gel. This was treated with pyridine (10 ml.) and acetic anhydride (15 ml.) to give a yellow solution, which was kept for 3 days at room temperature before being poured into water to yield acetate C as previously described (5·55 g.). $[\alpha]_D^9$ — 1·0° in chloroform (*c*, 1·01) (Found: Ac, 35·0%).

(2) *Laminarin benzoate.*—A monobenzoate of laminarin has been prepared by Connell *et al.*[1] by treating laminarin containing about 10% of moisture with pyridine and benzoyl chloride for 12 days at room temperature. Attempts have, therefore, been made to obtain a derivative with a higher benzoyl content. Preliminary experiments indicated that the conditions used for acetylation, particularly the reaction time, were unsuitable for benzoylation. The tribenzoate has been prepared as follows.

Laminarin (2·499 g.; 15·41 mg. mol. of $C_6H_{10}O_5$) was dissolved in water, precipitated with ethanol, and dispersed in pyridine (33 ml.) as described for acetate B. Benzoyl chloride (14·6 g.; 104 mg. mol.) was then added with cooling, when the solution turned pink and a precipitate formed. After 8 days at room temperature with mechanical shaking during 5 hours each day, the mixture was poured into water (250 ml.), when the benzoate separated as a dark red-brown oil. After being set aside overnight, the viscous syrup was centrifuged, stirred with hot water (3 × 150 ml.), and dried *in vacuo* over phosphorus pentoxide to a red-brown solid (6·952 g.). Yield, 95·0% assuming a tribenzoate (tribenzoyl derivative), $C_6H_7O_5Bz_3$, has been formed. The removal of colour from this crude product proved a difficult problem; solution in chloroform and precipitation with light petroleum removed very little colour, and fractionation of an acetone

solution with ether and then light petroleum was unsuccessful. Eventually, most of the colour was removed by dissolving the benzoate (6·537 g.) in acetone (130 ml.) and precipitating with water (160 ml.) containing a small quantity of sodium chloride to prevent the precipitate's becoming colloidal. This procedure was repeated three times, and the precipitate was finally dried *in vacuo* over phosphorus pentoxide to a cream-coloured powder (6·276 g.). Yield (from laminarin), 91·2%. $[\alpha]_D^{17}$ — 97·1° in chloroform (*c*, 1·01) (Found : C, 67·0 ; H, 4·78 ; Bz, 65·3%. Calc. for $C_6H_7O_5Bz_3$: C, 68·3 ; H, 4·67 ; Bz, 66·4%).

In determining the benzoyl content, tribenzoyl-laminarin was found to be unattacked by the conditions used for acetyl estimation. A slight modification of Mullen & Pacsu's method[14] for determining the acetyl value of starch acetate was employed. The benzoate (1 g.) was dissolved in acetone (25 ml.), 2N-sodium hydroxide (10 ml.) was added, and the solution was heated under reflux at 55–60° for one hour with frequent shaking. The brown solution was then diluted with water (500 ml.) and back-titrated with 0·5N-hydrochloric acid and phenolphthalein ; a control was also carried out. Even this drastic treatment sometimes failed to decompose the benzoate completely, and small particles of unchanged material were still visible.

Tribenzoyl-laminarin was soluble in chloroform, acetone, pyridine, benzene, tetrahydrofuran and ethyl acetate ; less soluble in glacial acetic acid ; and insoluble in water, ethanol, ether and light petroleum.

(3) *Laminarin carbanilate.*—Wolff & Rist[15] have shown that various polysaccharides, including corn and potato starch, glycogen and dextran, react with phenyl *iso*cyanate in the presence of pyridine to form tricarbanilates :

$$\underset{\text{Starch}}{C_6H_{10}O_5} + 3\underset{\substack{\text{Phenyl}\\ \textit{iso}\text{cyanate}}}{Ph{\cdot}NCO} \rightarrow \underset{\substack{\text{Starch}\\ \text{tricarbanilate}}}{C_6H_7O_5(CO{\cdot}NHPh)_3}$$

Laminarin readily formed a tricarbanilate in quantitative yield. Anhydrous laminarin (2·501 g.) was heated with pyridine (40 ml.) and phenyl *iso*cyanate (10 ml.) at 100° for 7½ hours with occasional stirring. After being set aside overnight at room temperature, the brown solution was centrifuged to remove a small residue, the supernatant liquid was poured into ethanol (200 ml.) to give a clear solution, and water (250 ml.) was added, when the ester appeared as a colloid. This was coagulated with saturated sodium chloride solution (6 ml.), and the precipitate was centrifuged and washed with water. The ester was purified by dissolving in acetone (50 ml.), adding ethanol (200 ml.) and precipitating with water (250 ml.) containing a small amount of sodium chloride. The tricarbanilate was centrifuged, washed with 50% (v/v) ethanol (2 × 100 ml.), and dried *in vacuo*, first over calcium chloride and finally over phosphorus pentoxide (8·219 g.). Yield (assuming a tricarbanilate), 103·5%. $[\alpha]_D^{20}$ — 6·9° in chloroform ; — 52·4° in pyridine (*c*, 1·01) ; m.p. 175–185° (without charring) (Found : ash, ± 0·0 ; C, 63·0 ; H, 5·30 ; N, 8·37%. Calc. for $C_6H_7O_5(CO{\cdot}NH{\cdot}Ph)_3$: C, 62·4 ; H, 4·85 ; N, 8·09%).

The product, which formed an almost white powder, was soluble in chloroform, pyridine, acetone, glacial acetic acid, ethyl acetate and tetrahydrofuran ; slightly soluble in ethanol ; and insoluble in water, ether, benzene, carbon tetrachloride and light petroleum.

(4) *Laminarin sulphate.*—Recently, Ricketts & Walton[16] have shown that low-molecular-weight dextran sulphates are active blood-anticoagulants. High-molecular-weight dextran was degraded by acid hydrolysis, and various dextran preparations containing a range of molecular weights were obtained by fractional precipitation. Dextran sulphate, with a molecular weight of about 7000, was found to be non-toxic, and maximum blood-anticoagulant activity was attained when the number of sulphate groups exceeded an average of 1·3 per glucose unit. It was considered that laminarin, in view of its low molecular weight, might prove successful as an anticoagulant, and consequently a series of laminarin sulphates have been prepared by the methods of Astrup *et al.*[17] and of Ricketts.[18] These are at present under test for their heparin activity. Sulphation is also an effective means of solubilizing insoluble laminarin, but these sulphates, in view of their possible anticoagulant activity, have not been investigated as substitutes for plasma.

By varying the proportion of chlorosulphonic acid in the reaction mixture, a series of laminarin sulphates have been prepared. A typical preparation is as follows : Chlorosulphonic acid (4·0 ml.) was added drop by drop to pyridine (40 ml.), which was cooled in ice–salt, and the mixture was then heated to 65°, when most of the white solid dissolved. Anhydrous, finely powdered laminarin (4·999 g.) was then added, and the mixture was heated at 65–70° for 30 minutes, with frequent stirring. After being cooled, the syrupy solution was dissolved in water (300 ml.), precipitated with ethanol (1000 ml.), and the viscous syrup centrifuged. The product was redissolved in water (150 ml.), neutralized to a phenolphthalein end-point with

2N-sodium hydroxide to decompose the pyridine salt, saturated sodium chloride solution (20 ml.) was added, and reprecipitation was carried out with ethanol (400 ml.). The syrup was dissolved in water (180 ml.), dialysed against running water until free from sulphate (2 days), and the solution was adjusted to pH 7·6 with sodium hydroxide and then concentrated *in vacuo* at 45° to 60 ml. Ethanol was added to give a concentration of 85% (v/v), and the precipitated syrup was centrifuged, washed with ethanol and ether, ground to a white powder, and dried *in vacuo* over phosphorus pentoxide (8·030 g.) [Found: ash (as Na_2SO_4), 32·3; i.e. SO_4, 21·8; total sulphate on hydrolysis (as SO_4), 43·5%].

The sulphate in the ash should be exactly one-half of the total sulphate for a polysaccharide sulphuric ester, for one-half of the sulphur is lost during ignition:

$$2(C_6H_9O_5{\cdot}SO_3Na) + 4H_2O \xrightarrow[\text{hydrolysis}]{\text{acid}} 2C_6H_{12}O_6 + H_2SO_4 + Na_2SO_4$$

Sodium laminarin sulphate — Glucose

$$2(C_6H_9O_5{\cdot}SO_3Na) \xrightarrow[\text{ashing}]{} Na_2SO_4 + SO_3 + \text{products of combustion}$$

The number of sulphate groups per glucose unit is calculated from the total sulphate content as follows:

$$\text{Let the repeating unit} = C_6H_{10-n}O_5(SO_3Na)_n$$

where n = number of sulphate groups per glucose unit.

Then the weight of repeating unit $= 162 - n + 103n = 162 + 102n$

and the total sulphate (as SO_4) $= \dfrac{96 \times n \times 100}{162 + 102n} = 43{\cdot}5$

Therefore $n = 1{\cdot}37$, and the weight of repeating unit = 302.

$$\%\ \text{Yield} = \frac{8{\cdot}030 \times 162 \times 100}{4{\cdot}999 \times 302} = 86{\cdot}2$$

A series of laminarin sulphates, with varying sulphate contents, have been prepared, and the results are recorded in Table III.

Table III

Analysis of various sodium laminarin sulphates

Laminarin sulphate	Chloro-sulphonic acid per 5 g. of laminarin, ml.	Reaction time at 65–70°, min.	Ash (as Na_2SO_4), %	Sulphate in ash (as SO_4), %*	Total sulphate on hydrolysis (as SO_4), %	No. of sulphate groups per glucose unit	Yield, %	Stability of 6% aqueous solution	$[\alpha]_D^{18}$ in water (c, 2·0)
A	8·8	75	41·6	28·1	54·1	2·15	99·4	no pptn. after 32 days	− 9·8°
B	6·0	30	41·7	28·2	52·9	2·04	104·6	—	—
C	4·0	30	32·3	21·8	43·5	1·37	86·2	no pptn. after 32 days	− 3·7°
D	2·0	30	19·6	13·2	26·1	0·61	87·1	18·2% pptd. after 11 days	− 8·2°
E	1·0	15	7·4	5·0	10·5	0·20	78·2	47·7% pptd. after 6 days	—

* Calculated from the ash

The results show that, for most samples, the total sulphate is very nearly twice the sulphate in the ash. Sulphates A, B, C and D are being tested as anticoagulants (patent applied for).

It is interesting to note that the insoluble material from sample D, which was precipitated from 6% aqueous solution after 11 days, contained an ash content of only 4·9%, although the ash in the original product was 19·6%. This suggests that sulphation is a random process

giving a series of molecules of widely varying sulphate content, and the calculated number of sulphate groups is merely an average; molecules with least sulphate attached have been precipitated from solution.

Summary

The solubilization of the cold-water-insoluble form of laminarin from *Laminaria cloustoni* frond has been investigated. Laminarin cannot be rendered soluble by modification of the terminal reducing groups at the end of the chains, either by oxidation with hypoiodite or hypochlorite or by reduction with sodium borohydride.

Various ethers of laminarin, however, have been prepared, aqueous solutions of which show no sign of precipitation on being set aside at room temperature for a period of several weeks. Hydroxyethyl- and hydroxypropyl-laminarin have been obtained in 88·3% and 75·9% yields by treating an alkaline solution of the polysaccharide with ethylene and propylene oxides respectively. Both derivatives are ash-free, like the original polysaccharide, the specific rotations are very little different from that of laminarin, and, being ethers, they should be just as stable to the action of acids and alkalis as laminarin itself. These are being tested as possible substitutes for blood plasma. Sodium carboxymethyl-laminarin, containing almost one carboxymethyl group per glucose unit, has been prepared in 94·7% yield. Its aqueous solution is stable at room temperature, but yields insoluble copper, silver, lead and aluminium salts. A water-insoluble benzyl ether, containing 0·5 benzyl groups per glucose unit, has also been prepared.

A number of esters of insoluble laminarin have been prepared, most of which are water-insoluble. Triacetyl-laminarin, $[\alpha]_D^{17}$ — 63·6° in chloroform, has been obtained in 88·3% yield by treating the freshly precipitated polysaccharide with acetic anhydride and pyridine in the cold for 2 days. An acetate with a lower acetyl content, $[\alpha]_D^{9}$ — 1·0° in chloroform, was obtained when the laminarin was dispersed in pyridine by the Pacsu & Mullen method.[13] Benzoyl chloride and pyridine, after 8 days at room temperature, gave the tribenzoyl ester in 91·2% yield, and phenyl *iso*cyanate and pyridine at 100° for 7½ hours gave the tricarbanilate in quantitative yield. Laminarin triacetate, tribenzoate and tricarbanilate are insoluble in water, but are soluble in a wide range of organic solvents.

Laminarin has been sulphated with chlorosulphonic acid and pyridine, and a series of sulphates, containing from 2·15 to 0·20 sulphate groups per glucose unit, have been prepared. Those containing 2·15 and 1·37 sulphate groups per glucose unit gave 6% aqueous solutions which showed no sign of precipitation after being set aside for 32 days. It was considered that laminarin sulphate might possess anticoagulant properties similar to those of dextran sulphate and other sulphated polysaccharides, and these esters are being tested for heparin activity.

Acknowledgment

The work described in this paper forms part of the programme of research and development on seaweed undertaken by the Institute of Seaweed Research, and the authors are indebted to the Director for permission to publish.

Institute of Seaweed Research
Inveresk
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Received 12 October, 1953

References

1 Connell, J. J., Hirst, E. L., & Percival, E. G. V., *J. chem. Soc.*, 1950, p. 3494
2 Black, W. A. P., Cornhill, W. J., Dewar, E. T., & Woodward, F. N., *J. appl. Chem.*, 1951, **1**, 505
3 Barry, V. C., *Sci. Proc. R. Dublin Soc.*, 1939, **22**, 59
4 Degering, E. F., 'Chemistry and Industry of Starch' (Ed. R. W. Kerr), 1950, 2nd edn., p. 259 (New York: Academic Press Inc.)
5 Corbett, W. M., Kenner, J., & Richards, G. N., *Chem. & Ind.*, 1953, p. 462
6 Blair, M. G., & Reeves, R. E., *J. Amer. chem. Soc.*, 1952, **74**, 2622
7 Craik, J., *J. Soc. chem. Ind., Lond.*, 1924, **43**, 171T
8 Abdel-Akher, M., Hamilton, J. K., & Smith, F., *J. Amer. chem. Soc.*, 1951, **73**, 4691
9 Ziese, W., *Hoppe-Seyl. Z.*, 1935, **235**, 235
10 Chowdhury, J. K., *Biochem. Z.*, 1924, **148**, 76
11 Gomberg, M., & Buchler, C. C., *J. Amer. chem. Soc.*, 1921, **43**, 1904
12 Percival, E. G. V., & Ross, A. G., *J. chem. Soc.*, 1951, p. 720
13 Pacsu, E., & Mullen, J. W., *J. Amer. chem. Soc.*, 1941, **63**, 1487
14 Mullen, J. W., & Pacsu, E., *Industr. Engng Chem. (Industr.)*, 1942, **34**, 1209
15 Wolff, I. A., & Rist, C. E., *J. Amer. chem. Soc.*, 1948, **70**, 2779
16 Ricketts, C. R., & Walton, K. W., *Chem. & Ind.*, 1952, p. 869
17 Astrup, T., Galsmar, B., & Volkert, M., *Acta physiol. scand.*, 1944, **8**, 215
18 Ricketts, C. R., *Biochem. J.*, 1952, **51**, 129

Printed in Great Britain by Butler & Tanner Ltd., Frome and London

The use of charcoal columns for demineralizing mannitol–salt mixtures and for preparing laminaribiose from laminarin has also been investigated.

Experimental

Species examined

The composition of the samples of the two species of seaweed used in these investigations is recorded in Table I.

Table I

Analysis of seaweed species examined

Species	% Chemical composition (dry basis)						
	Laminarin	Fucose (as $C_6H_{12}O_5$)	Mannitol	Ash	Alginic acid	Cellulose	Organic N
Fucus vesiculosus (Fv/XII/45/ME)	2·6	9·20	9·0	26·0	15·0	—	1·20
Laminaria cloustoni (C/X/48) frond	29·2	3·14	15·4	20·3	10·0	5·4	1·70

Unless otherwise stated, all rotations were measured in a 2-dm. tube, and all paper chromatograms were run in *n*-butanol–ethanol–water (40 : 11 : 19 by vol.) and sprayed with benzidine–trichloroacetic acid.[8]

Separation of glucose–fucose mixtures on charcoal–Celite

(1) *Using charcoal–Celite column* (46 × 5·0 *cm.*).—The column was prepared as described by Whelan, Bailey & Roberts.[8] To prevent the growth of moulds when not in constant use, the column was washed through with 0·1% mercuric chloride solution after each experiment. Organic solvents were avoided (except in the preparation of laminaribiose) because aqueous ethanol is difficult to remove even on prolonged washing with water[9] and it also alters the characteristics of the column.

Preliminary experiments showed that it was impossible to separate a mixture of glucose, fucose and mannitol (500 mg. of each) on this column, using water as the eluant. Glucose and mannitol emerged from the column almost at the same time. A mixture of glucose and fucose, however, has been successfully separated as described below.

D-Glucose (997 mg.) and L-fucose (986 mg.) were dissolved in water (20 ml.), the solution added to the column, and the column eluted with water under gentle suction at the rate of 200 ml./h. Fifty-ml. fractions were collected and the elution of the sugars was followed by measuring the optical rotation (α_D) of the fractions (Table II).

Table II

Optical rotation of the fractions of eluate

Fraction No.	1–18	19	20	21	22	23	24	25	26	27–34	35
α_D	0·00	0·08	0·32	0·85	0·66	0·14	− 0·19	− 0·49	− 0·62	N.D.	− 0·01

N.D. = not determined

Appropriate fractions were then combined as shown in Table III, concentrated to dryness at 45° under reduced pressure, dried with ethanol–benzene, weighed and the specific rotations determined. The composition of the syrups was calculated from the rotations, taking the equilibrium $[\alpha]_D$ of D-glucose as + 52·7° and that of L-fucose as − 76·0° in water.

These results show that more than 90% of each sugar has been separated in a high degree of purity. The facts that the total weight of syrup recovered (column 2) is slightly more than that of the starting material, and that both the glucose and fucose balances (columns 7 and 8) exceed 100%, can be attributed to the presence of a small amount of moisture in the dried fractions.

The rate of elution was found to be an important factor in obtaining pure fractions. When the above experiment was repeated with an elution rate of 500 ml./h., 82·0% of the glucose was

separated in 98·1% purity, but the fucose syrup was badly contaminated with glucose (0·927 g.; $[\alpha]_D^{19}$ — 54·8°, i.e., fucose, 83·5%; glucose, 16·5%), and this was confirmed by chromatographic analysis.

The above results show that glucose is eluted by a much smaller volume of water than is fucose, which tends to have a long 'tail'; but it is advisable not to introduce ethanol into the eluant for the reasons mentioned above.

Table III

Fraction No.	Wt. of dried syrup (mg.)	$[\alpha]_D^{16}$ in water	Concentration *c*	% Chemical composition		Glucose separated as % of total glucose	Fucose separated as % of total fucose
				Glucose	Fucose		
19–22	914	+ 52·0°	1·828	99·4	0·6	91·1	0·6
23	145	+ 23·8	0·725	77·6	22·4	11·3	3·3
24	79	— 46·2	0·395	23·1	76·9	1·8	6·2
25–35	917	— 72·5	1·834	2·7	97·3	2·5	90·5

(2) *Using charcoal–Celite column* (113 × 7·3 *cm.*).—Three experiments have been carried out with this larger column, which has approximately five times the volume of the previous one, to compare the efficiencies of the two columns. In experiment A, glucose (5·012 g.) and fucose (5·020 g.) were placed on the column and eluted with water at the same rate as in the small-scale experiment, viz. 200 ml./h. Fifty-ml. fractions were collected as before and the elution of the sugars was followed polarimetrically as shown in Fig. 1A. The rotation of the combined dextrorotatory fractions (112–151) showed a 99·9% recovery of D-glucose, indicating a complete separation of the two sugars. In experiment B, the quantity of each sugar was doubled but the elution rate was kept constant at 200 ml./h. Part of the elution curve is shown in Fig. 1B and the results are summarized in Table IV. Separation of 79·5% of the glucose in 97·3% purity was effected, but the fucose fraction was impure. The low recovery of fucose (78·6%) is due to the incomplete elution of this sugar from the column; fraction 225 still possessed α_D of — 0·07°. Very large volumes of water are required with this column to elute completely all the fucose present.

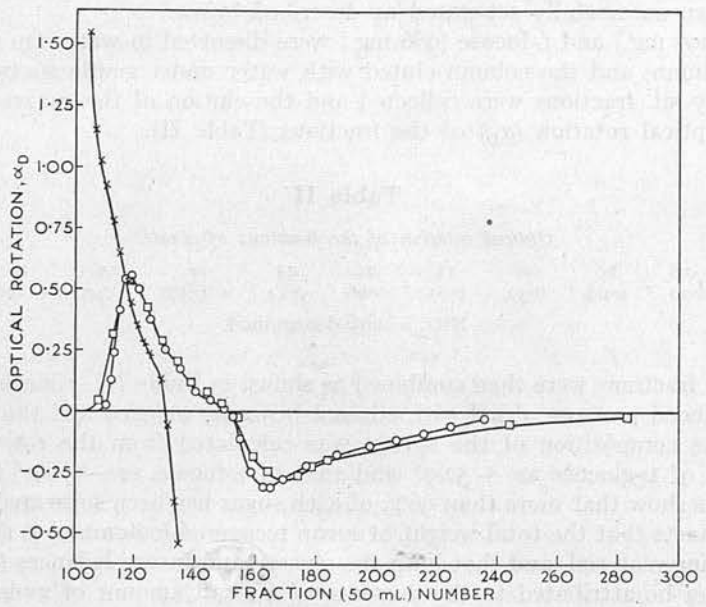

FIG. 1.—*Elution curves for experiments A, B and C*

○—○—○ Experiment A
×—×—× Experiment B
□—□—□ Experiment C

Table IV

Composition of the syrups obtained in experiments B and C

Experiment No.	Wt. of glucose added (g.)	Wt. of fucose added (g.)	Rate of elution (ml./h.)	Total volume of combined fractions (ml.)	Wt. of syrup recovered (g.)	$[\alpha]_D$ in water	Concentration, *c*
B	10·00	9·910	200	1200	8·175	+ 49·3°	1·872
				200	0·339	+ 16·2	0·678
				4700	9·328	− 50·4	1·794
C	5·00	4·971	1000	2000	4·336	+ 52·0	1·752
				500	0·199	+ 6·0	0·994
				6450	4·364	− 61·3	1·345

Experiment No.	Fractions (50 ml.) combined	% Chemical composition		Glucose separated as % of total glucose	Fucose separated as % of total fucose
		Glucose	Fucose		
B	104–127	97·3	2·7	79·5	2·2
	128–131	71·6	28·4	2·4	1·0
	132–225	19·9	80·1	18·6	75·4
C	107–146	99·4	0·6	86·2	0·5
	147–156	63·7	36·3	2·5	1·5
	157–285	11·4	88·6	9·9	77·8

In experiment C, 5 g. of each sugar were eluted at 1000 ml./h., so that this experiment is comparable with the small-scale one described under (1). Because of the difficulty of removing traces of moisture from these syrups, concentrations of solutions after measurement of specific rotation were determined in this case by evaporating aliquot portions (containing about 200 mg. dry matter) and drying at 100° for 16 h.; this treatment leads to a slight decomposition of the syrups, particularly where the fucose content is high, so that the resulting specific rotations may be slightly too high in this experiment. The results in Table IV show that 86·2% of the glucose can be isolated in the pure state, but the fucose is contaminated with glucose. It would appear, therefore, that this larger-scale experiment is not so efficient as that described under (1). Again, fucose was still being eluted from the column at fraction 285 (Fig. 1C).

Application of the method to hydrolysed Laminaria cloustoni *frond*

Treatment of a brown seaweed with N-sulphuric acid at 100° would be expected to hydrolyse the laminarin to D-glucose and the fucoidin to L-fucose, but to have little or no effect on the alginate or cellulose present, whilst the mannitol and salts would be brought into solution. After removal of the weed residue, therefore, the resulting hydrolysate should contain glucose, fucose, mannitol and salts, together with small quantities of other sugars, e.g., xylose, galactose and mannose,[10] which are also liberated on hydrolysis. Mannitol was eliminated by extracting the weed before hydrolysis with 85% methanol. It is necessary to use aqueous methanol, as anhydrous methanol fails to remove all the mannitol.

The method has been used to isolate glucose from *Laminaria cloustoni*. The dried, milled frond (3·116 g.) was refluxed three times with 85% (v/v) methanol (100 ml. each time) for 2 h., and then centrifuged and air-dried. It was hydrolysed with N-sulphuric acid (50 ml.) at 100° for 4 hr., the weed residue centrifuged, washed with water, the centrifugate and washings de-ionized with Zeo-Karb 225 and Amberlite IR-4B(OH), and the resulting solution concentrated *in vacuo* to dryness. The syrup (1·177 g.) was dissolved in water (20 ml.), the solution added to the column (46 × 5·0 cm.) and the column eluted with water (200 ml./h.) as previously described (Table V).

With the smaller quantity of fucose present (98 mg.), the separation is more clear-cut, fractions 33–35 (150 ml.) containing no sugars. Glucose does not emerge until later and the

glucose ' band ' is more diffuse (fractions 22–32). Fractions 23–30 were concentrated to a syrup (0·855 g.), $[\alpha]_D^{18} + 49{\cdot}0°$ in water (*c*, 1·71), i.e., glucose, 92·9% (assuming impurities are inactive). Yield from the original frond, 78·5%.

Chromatography showed the complete absence of fucose in the syrup, although a trace of an unknown ($R_{glucose}$ 0·43) was detected. In view of the small quantity present (98 mg.), no attempt was made to isolate the fucose from the other fractions.

Table V

Optical rotation of eluate fractions (50 *ml.*)

Fraction No.	1–21	22	24	26	28	30
α_D	0·00	0·02	0·14	0·45	0·27	0·11
Fraction No.	32	33–35	36	38	40	42
α_D	0·02	0·00	− 0·01	− 0·02	− 0·03	− 0·02

Use of charcoal–Celite for removing salts from solution

Whelan *et al.*[8] found that sodium sulphate was eluted before glucose on a column, and suggested the use of charcoal for de-ionizing purposes. The technique has now been used for demineralizing mannitol–salt mixtures (see below), and has also been applied to seaweed hydrolysates. Dried, milled *Laminaria cloustoni* frond (3·132 g.) was extracted with 85% methanol and hydrolysed with N-sulphuric acid (50 ml.) as previously described. The hydrolysate was neutralized with 2N-sodium hydroxide, concentrated to 20 ml., and the solution fractionated on the column (46 × 5·0 cm.). Glucose was eluted in the same position as in the previous experiment (Table V). Fractions 8–22 (zero rotation) were evaporated to a mass of white salts (3·70 g. ; 50 ml. of N-Na_2SO_4 contain 3·55 g. of anhyd. Na_2SO_4). Fractions 23–30 yielded a colourless syrup (0·961 g.), $[\alpha]_D^{17} + 45{\cdot}9°$ in water (*c*, 1·922), i.e., glucose, 87·1% (assuming impurities are inactive). Yield from the original frond, 82·4%.

The glucose syrup in this case is less pure than previously, but the purity is probably sufficiently high to allow crystallization from water on a larger scale. Chromatography (*tert.*-amyl alcohol–*n*-propanol–water 40 : 10 : 15 by vol.)[11] showed a trace of xylose in addition to glucose and the unknown, while the periodate–benzidine spray[11] also revealed the presence of mannitol, indicating that even 85% methanol does not remove all the mannitol from the frond.

Application of the method to Fucus vesiculosus

This alga contains xylose, 1·1%, and galactose, 0·4%, and a trace of mannose,[10] in addition to the constituents shown in Table I. It also yields, on hydrolysis, a considerable amount of a glassy material which does not move on a cellulose column[10] but which is eluted with water on a charcoal column in the same position as hexoses. Although it has not been examined in detail, this material is soluble in water, laevorotatory, insoluble in ethanol, and one sample gave ash, 12·1%, and $[\alpha]_D^{17} - 12°$ (water). *Laminaria cloustoni* frond did not give this material to anything like the same extent.

In spite of these difficulties, fucose has been isolated from *Fucus vesiculosus* in a fair degree of purity on the small scale. Dried, milled weed (10·05 g.) was refluxed three times with 85% methanol (120 ml. each time for 2 h.), hydrolysed with N-sulphuric acid (100 ml.) at 100° for 4 h., the hydrolysate and washings neutralized with 2N-sodium hydroxide, and concentrated *in vacuo* to 40 ml. After removal of a residue by filtering, the solution was added to the column (46 × 5·0 cm.), which was eluted with water (200 ml./h.). The elution of the sugars was followed polarimetrically (Table VI).

Table VI

Optical rotation of eluate fractions (50 *ml.*)

Fraction No.	1–22	23	25	27	29	31	33	35	41	48	50
α_D (4-dm. tube)	0·00°	− 0·06	− 0·26	− 0·12	− 0·06	− 0·14	− 0·75	− 0·73	− 0·30	− 0·06	− 0·03

The ' glassy material ' was responsible for the negative rotation up to fraction 30, after which fucose was eluted as evidenced by the much stronger laevorotation. Fractions 23–30 were combined to give a white glass (1·255 g. ; 12·5% of the alga), which was not further examined. Fractions 31–50 on concentration yielded a syrupy glass (1·381 g.), which was extracted with cold ethanol (2 × 25 ml.), and the extracts centrifuged to remove a white residue. The centrifugate was then concentrated to a syrup (0·889 g.), $[\alpha]_D^{18}$ — 67·5° in water (*c*, 1·778). Chromatography revealed the presence of fucose with traces of xylose, mannose, galactose and an unknown (R_F less than that of galactose). The syrup (0·860 g.), however, readily crystallized from ethanol (2 ml.) to give L-fucose (0·593 g.). Equilibrium $[\alpha]_D^{17}$ — 71·9° in water (*c*, 2·46), i.e., 94·5% L-fucose (assuming impurities are inactive) ; yield from the original seaweed, 62·7%.

The purity of the sugar compares favourably with that obtained by previous methods[1, 2] after one crystallization, while the yield is considerably improved.

However, when attempts were made to increase the quantity of fucose from 1 g. to about 5 g. on the same column, serious difficulties were encountered. Fucose began to emerge about ten fractions (500 ml.) in advance of its normal position, so that the separation of fucose from the other hexoses and xylose was less effective. This is illustrated by the following experiment :

The seaweed (50·55 g.) was extracted with 85% methanol, hydrolysed (500 ml. N-H_2SO_4), and the hydrolysate neutralized as described above. Since 500 ml. of N-sulphuric acid on neutralization give 35·5 g. of sodium sulphate, the bulk of this salt was precipitated with ethanol to avoid overloading the column with mineral matter ; in addition, ethanol removed a large proportion of ' glassy material '. The solution was, therefore, concentrated to 100 ml., ethanol (1 l.) added, the bulky precipitate centrifuged, and the centrifugate evaporated *in vacuo* to a brown mixture of sugars and salts (7·95 g.), which was then fractionated on the column (46 × 5·0 cm. ; 200 ml./h.). The following rotations were observed :

Table VII

Optical rotation of eluate fractions (50 *ml.*)

Fraction No.*	1–16	16½	17	17½	18	18½	19	19½
α_D	0·00°	0·00	— 0·02	— 0·13	— 0·17	+ 0·23	+ 0·48	— 0·35
Fraction No.*		20	20½	21	23	28	43	44
α_D		— 1·45	— 1·99	— 2·08	— 1·78	— 0·67	— 0·02	N.D.

* 16½ to 21 represent 25-ml. fractions

Fractions 17–18 contained ' glassy material ' and were rejected. Fractions 18½–19 yielded a colourless syrup (0·721 g.), chromatography of which showed xylose (strong), galactose/glucose (medium), an unknown (medium ; R_F less than that of galactose), mannose (trace) and fucose (trace). A solution of this syrup in water (14 ml.), when treated with ethanol (14 ml.), methylphenylhydrazine (1·4 ml.) and acetic acid (0·3 ml.) at 2° for 2 days, yielded galactose methylphenylhydrazone (0·139 g.) ; m.p. 183–184°, after recrystallization from ethanol ; mixed m.p. with D-galactose methylphenylhydrazone, 184–185°. This confirms the presence of galactose in *Fucus vesiculosus*.[10]

The fucose-containing fractions 20–44 were concentrated to a syrupy glass (5·55 g.), $[\alpha]_D^{17}$ — 56·3° in water (*c*, 2·22). This was extracted as before with cold ethanol (150 ml.), insoluble ' glassy material ' ($[\alpha]_D^{17}$ — 12° in water ; *c*, 0·925 ; ash, 12·1%) centrifuged off, and the centrifugate concentrated to a syrup (5·060 g.), the chromatography of which showed fucose (very strong), xylose (medium), galactose/glucose (weak), an unknown (trace ; R_F less than that of galactose) and mannose (trace). Dissolution of the syrup in ethanol (10 ml.) at 2° for 2 days gave the crystalline sugar (3·981 g.), which, however, was still impure. Equilibrium $[\alpha]_D^{16}$ — 63·0° in water (*c*, 2·222), i.e., 82·9% L-fucose (Found : ash, 1·0%).

These results show that the separation of the various sugars at this concentration is not sufficiently clear-cut to give a pure fucose fraction. With more than 1 g. of fucose in solution, the fucose ' band ' moves forward so that it becomes contaminated with other monosaccharides. It is doubtful, therefore, if this method could compete with previous methods[1, 2] for the large-scale preparation of fucose.

Demineralization of mannitol–salt mixtures

Mannitol–salt mixtures, obtained by aqueous acid extraction of seaweed samples followed by removal of laminarin and fucoidin by alcohol precipitation,[12] can be partially demineralized on charcoal–Celite, and this affords another method of isolating mannitol from these mixtures. The results in Table VIII were obtained with a mannitol (38·7%)–salt (37·9%) mixture (prepared from *Laminaria cloustoni* frond as previously described[12]) on the column (46 × 5·0 cm.). In both cases the mixture was dissolved in water (40 ml.), the solution added to the column and eluted with water at 200 ml./h., mannitol being detected by spotting on filter paper and spraying with periodate–benzidine reagent.[11] The fractions containing mannitol were combined to give

Table VIII

Demineralization of mannitol (38·7%)*–salt* (37·9%) *mixture*

Expt. No.	Wt. of mixture taken (g.)	Mannitol-containing fractions (50 ml.)	Wt. of crude mannitol (g.)	Analysis of crude mannitol		
				Ash %	Mannitol %	Mannitol as % of mannitol in mixture
1	14·64	20–31	6·75	10·5	84·6	100·8
2	29·93	18–19	5·787	38·3	38·4	19·2
		20–27	12·10	18·2	74·3	77·6

crude mannitol as a white crystalline solid. In experiment 1, the salt-containing fractions (8–19) were evaporated to yield a white mass of salts (5·60 g.), which gave an ash content of 91·0%, i.e., 91·9% of total ash in the original mixture.

The results show that the mineral content of mannitol–salt mixtures can be considerably reduced in this way, and pure mannitol can readily be obtained from the crude product by recrystallization from water. In experiment 2, the large amount of salt present (11·34 g.) rendered the separation less clear-cut, and the crude mannitol still possessed a high ash content (18·2%).

It has been found, however, that pure mannitol can be prepared from mannitol–salt mixtures, containing more or less any ratio of mannitol to salt, by direct crystallization from water, thereby eliminating the use of charcoal columns or any other method of separation.[12] This method is based on a Japanese patent.[13] The mannitol–salt mixture was extracted at 90° with water equal in weight to the mannitol present in the mixture, the undissolved salts filtered off from the hot solution, and the filtrate allowed to crystallize. The above mannitol (38·7%)–salt (37·9%) mixture (25·35 g.) was heated with water (10 ml.) at 90° and the salts filtered off, when the filtrate crystallized on cooling. After 16 h. at 2°, the crude mannitol was filtered, washed with saturated mannitol solution (5 ml.), ethanol and ether, and dried at 100° (6·049 g.). Found: ash, 10·1; mannitol, 90·9%. Yield, 56·0%. This crude material (5·628 g.), on recrystallization from water (5 ml.), gave pure mannitol (4·406 g.). Found: ash, 0·15%; m.p. 165–167°. Yield from the original mixture, 48·2%.

In both the direct crystallization method and the charcoal–Celite column, it is essential that the laminarin and fucoidin are first removed from the aqueous extract of the weed by alcohol precipitation. The presence of polysaccharides in the direct crystallization method makes the extract of the mannitol–salt mixture so viscous that it is impossible to remove the undissolved salts by filtration, while with charcoal–Celite, laminarin and fucoidin contaminate the column and are not readily eliminated afterwards. It has been found unnecessary in the preparation of mannitol–salt mixtures to raise the concentration of alcohol to 85% (w/w) to remove polysaccharides as previously described.[12] The dilute aqueous acid extract of the seaweed is neutralized, concentrated under reduced pressure to a viscous solution containing 10–15% (w/v) of mannitol, and ethanol or methanol added to 80% (v/v). The precipitated material, containing laminarin, fucoidin and some of the salts, e.g., sulphates, is then centrifuged, and the centrifugate evaporated to give the mannitol–salt mixture as a light-green or brown solid.

Preparation of laminaribiose from laminarin

The disaccharide, laminaribiose, was first isolated by Barry,[14] who heated laminarin with N-oxalic acid until the hydrolysis was about two-thirds complete (7 h., 100°), and then destroyed

the glucose with yeast. The optically active liquid remaining was dissolved in methanol, and the laminaribiose was obtained as a powder by fractional precipitation with ethanol and ether. Connell, Hirst & Percival[15] also obtained the disaccharide by partial hydrolysis of laminarin with oxalic acid and separation of the sugars on a cellulose column. Proof of the structure of laminaribiose as 3-*O*-β-D-glucopyranosyl-D-glucose has been obtained by its synthesis from 1 : 2-5 : 6-di-*O*-*iso*propylidene-glucose and tetra-*O*-acetylglucosyl bromide.[16, 17] Peat, Whelan & Lawley[18] used charcoal–Celite columns for separating laminaribiose, gentiobiose, $\beta\beta$-trehalose and five trisaccharides from a partial hydrolysate of insoluble laminarin. Recently, they have shown that the material thought to be $\beta\beta$-trehalose is, in fact, 1-β-glucosyl-mannitol.[19]

Laminaribiose can readily be prepared from laminarin by the use of charcoal–Celite columns. Laminarin (25·02 g.; from *Laminaria cloustoni* frond) was partially hydrolysed with 0·25N-sulphuric acid at 100° for 5 h. (% hydrolysis, 52·2);[7] the hydrolysate was centrifuged to remove a small residue, and the solution was neutralized with Amberlite resin IR-4B(OH) and concentrated at 45° under reduced pressure. The resulting syrup (28·20 g.) was dissolved in water (40 ml.), the solution added to the column (46 × 5·0 cm.) and the column eluted as described by Whelan *et al.*[8] in their preparation of the maltodextrins. Fractions of 150 ml. were collected and the elution of the sugars with water and 7·5% ethanol was followed polarimetrically as shown in Fig. 2. The rotation of the combined dextrorotatory fractions (7–11) obtained with water as eluant showed 13·2 g. (47·5% yield from laminarin) of glucose present (13·5 g. by hypoiodite estimation[20]).

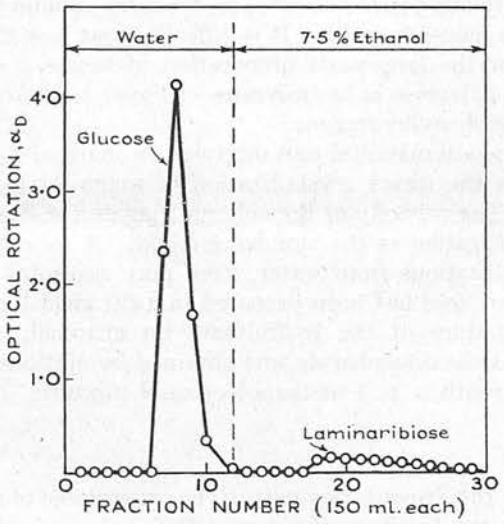

FIG. 2.—*Chromatographic separation of glucose and laminaribiose on charcoal*

The disaccharide fractions (18–30) obtained with 7·5% ethanol were evaporated to a glass (5·10 g.), $[\alpha]_D^{12} + 19{\cdot}9°$ in water (*c*, 1·205). Chromatography (*n*-butanol–pyridine–water–benzene 5 : 3 : 3 : 1 by vol.) showed laminaribiose ($R_{glucose}$ 0·69) and an unknown ($R_{glucose}$ 0·29), but glucose was completely absent. The disaccharide was crystallized by dissolving the glass (4·40 g.) in anhydrous methanol (30 ml.) and adding ethanol (30 ml.), when crystals formed almost immediately. After 3 h. at 2°, the first crop (0·189 g.) was filtered off and rejected. The filtrate, after 7 days at 2°, gave crystalline laminaribiose, which was filtered, washed with ethanol (5 ml.) and dried *in vacuo* over phosphorus pentoxide to a white powder (3·234 g.). Yield, 14·2% (from laminarin); $[\alpha]_D^{20} + 22{\cdot}4°$ (4 min.) $\rightarrow$ + 17·8° in water (*c*, 2·0); Bächli & Percival[17] quote $[\alpha]_D^{16} + 24{\cdot}9°$ (20 min.) $\rightarrow$ + 18·6° in water for synthetic α-laminaribiose; m.p. 198–201° (uncorr.). Found: ash, ± 0·0; C, 41·9; H, 6·6. Calc. for $C_{12}H_{22}O_{11}$: C, 42·1; H, 6·5%. Estimations by hypoiodite oxidation,[20] however, were invariably low (92·0 — 92·4%) for this disaccharide. Chromatography (*n*-butanol–pyridine–water–benzene 5 : 3 : 3 : 1) showed a single spot.

Summary

(1) A charcoal–Celite column (46 × 5·0 cm.) can effectively separate a mixture of 1 g. of glucose and 1 g. of fucose, using only water as the eluant; more than 90% of each sugar was recovered in 97–99% purity.

A column (113 × 7·3 cm.) of approximately five times the volume will separate a mixture of 5 g. of glucose and 5 g. of fucose, when the rate of elution with water (200 ml./h.) is kept the same as on the small scale. When the elution rate is increased five times (1000 ml./h.), the fucose becomes contaminated with glucose.

(2) D-Glucose has been isolated on the 1-g. scale, in 78% yield and 93% purity, from hydrolysed *Laminaria cloustoni* frond, from which mannitol had previously been eliminated by 85%-methanol extraction. The separation from fucose was complete, although the sugar was slightly contaminated with traces of unidentified material.

When the column was used both as a means of separating the sugars and for demineralization, glucose was isolated in 82% yield and 87% purity.

(3) L-Fucose has been isolated on the 1-g. scale, in 63% yield and 94% purity, from methanol-extracted, hydrolysed *Fucus vesiculosus*. This compares favourably with previous methods for obtaining fucose from seaweeds.

Hydrolysates of *Fucus vesiculosus* contain, however, in addition to fucose and glucose, various other constituents, e.g., xylose, galactose and a laevorotatory, unidentified 'glassy material', which interfere with the separation of fucose on charcoal–Celite columns. When the experiment was scaled up to the 5-g. fucose level on the same column (46 × 5·0 cm.), it was not possible to separate a pure fucose fraction. It is difficult to see how the method could compete with previous methods for the large-scale preparation of fucose.

(4) The presence of D-galactose in hydrolysates of *Fucus vesiculosus* has been confirmed by the isolation of its methylphenylhydrazone.

(5) The demineralization of mannitol–salt mixtures on charcoal–Celite has also been investigated and compared with the direct crystallization of mannitol from concentrated aqueous mannitol–salt solutions. The capacity of the column is again the limiting factor, and it would appear that direct crystallization is the simpler method. A mannitol (38·7%)–salt (37·9%) mixture, after two crystallizations from water, gave pure mannitol in 48% yield.

(6) Crystalline laminaribiose has been prepared in 14% yield by the partial hydrolysis of laminarin and chromatography of the hydrolysate on charcoal. Glucose was completely eliminated with water, and the disaccharide was obtained by elution with 7·5% ethanol. The sugar readily crystallized with a 1 : 1-methanol–ethanol mixture.

Acknowledgments

The work described in this paper forms part of the programme of research and development on seaweed undertaken by the Institute of Seaweed Research, and the authors are indebted to the Institute for permission to publish. They are also indebted to Dr. W. J. Whelan, University College of North Wales, Bangor, for help and advice on the use of charcoal columns, particularly with the early work on pure glucose–fucose mixtures.

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Received 14 April, 1955

References

[1] Bates, F. J., & Associates, Polarimetry, Saccharimetry and the Sugars, Circular C440 of the National Bureau of Standards, 1942, p. 460 (Washington, D.C.: U.S. Govt. Printing Office)

[2] Black, W. A. P., Cornhill, W. J., Dewar, E. T., & Woodward, F. N., *J. Sci. Fd Agric.*, 1953, **4**, 85

[3] Black, W. A. P., Dewar, E. T., & Woodward, F. N., *J. Sci. Fd Agric.*, 1952, **3**, 122

[4] Whelan, W. J., & Morgan, K., *Chem. & Ind.*, 1954, p. 78

[5] Lindberg, B., & Wickberg, B., *Acta chem. scand.*, 1954, **8**, 569

[6] Hough, L., Jones, J. K. N., & Wadman, W. H., *J. chem. Soc.*, 1949, p. 2511

[7] Black, W. A. P., Dewar, E. T., & Woodward, F. N., *J. Sci. Fd Agric.*, 1953, **4**, 58

References—*contd.*

[8] Whelan, W. J., Bailey, J. M., & Roberts, P. J. P., *J. chem. Soc.*, 1953, p. 1293
[9] Whelan, W. J., private communication
[10] Dewar, E. T., *Chem. & Ind.*, 1954, p. 785
[11] Cifonelli, J. A., & Smith, F., *Analyt. Chem.*, 1954, **26**, 1132
[12] Black, W. A. P., Dewar, E. T., & Woodward, F. N., *J. appl. Chem.*, 1951, **1**, 414
[13] Sagawa, K., Sakai, T., & Miyagawa, M., Jap. Pat. 832 (1953)
[14] Barry, V. C., *Sci. Proc. R. Dublin Soc.*, 1941, **22**, 423
[15] Connell, J. J., Hirst, E. L., & Percival, E. G. V., *J. chem. Soc.*, 1950, p. 3494
[16] Freudenberg, K., & Oertzen, K., *Liebigs Ann.*, 1951, **574**, 37
[17] Bächli, P., & Percival, E. G. V., *J. chem. Soc.*, 1952, p. 1243
[18] Peat, S., Whelan, W. J., & Lawley, H. G., *Biochem. J.*, 1953, **54**, xxxiii
[19] Peat, S., Whelan, W. J., & Lawley, H. G., *Chem. & Ind.*, 1955, p. 35
[20] Hirst, E. L., Hough, L., & Jones, J. K. N., *J. chem. Soc.*, 1949, p. 928

Reprinted from the Journal of the Science of Food and Agriculture, 1951, No. 9, pp. **387–390.**

A METHOD FOR THE ESTIMATION OF FUCOSTEROL IN SEAWEEDS

By W. A. P. BLACK and W. J. CORNHILL

A method has been worked out for the estimation of fucosterol in seaweed samples. Ether has been found to be the most satisfactory solvent for the extraction of the fats; after saponification, the fucosterol is estimated in the unsaponifiable fraction by a colorimetric method based on the Liebermann–Burchard colour reaction.

Introduction

Fucosterol, which was first isolated from the brown marine alga (*Fucus vesiculosus*) by Heilbron, Phipers & Wright (1934), who showed it had the empirical formula $C_{29}H_{48}O$, may have some potential value as a source for the preparation of vitamin-D-active compounds and sex hormones. These workers showed that it was a bond isomer of stigmasterol, and as treatment with ozone failed to give ethyl*iso*propylacetaldehyde they concluded that the double bonds were in the nucleus. Later it was proved that one of the ethylenic linkages occupied the 5 : 6-position (Coffey, Heilbron, Spring & Wright, 1935; Coffey, Heilbron & Spring, 1936).

The presence of fucosterol in other brown algae was later demonstrated by Carter, Heilbron & Lythgoe (1939).

A sterol isolated from several Japanese algae by Sirahama (1942) was called pelvesterol, but its chemical properties agreed with those of fucosterol prepared by Coffey *et al.* (1935, 1936). In experiments on its activating influence on lipase, pelvesterol had three times the activating capacity of cholesterol, phytosterol or ergosterol.

The position of the second double bond remained in doubt until MacPhillamy (1942) showed that it was located in the side chain between carbon atoms 24 and 28. This work has been confirmed by Hey, Honeyman & Peal (1950), who also succeeded in reducing the 24-keto-cholesterol obtained on ozonolysis to cholesterol.

Although many procedures are available for the determination of cholesterol in blood and animal tissue and for the total sterols in vegetable oils and plant leaves, no specific method has been worked out for the estimation of fucosterol. Cholesterol has been estimated by colorimetric means for many years and there are numerous references in the literature especially in the field of blood chemistry. The method used depends on the colour based on the Liebermann–Burchard reaction for cholesterol, and the application of this method to fucosterol has now been studied.

Experimental procedure

Reagents

Acetic anhydride/concentrated sulphuric acid.—1 vol. sulphuric acid (A.R., sp. gr. 1·84) added slowly to 10 vol. acetic anhydride (A.R.) cooled in water.

Ethanolic potassium hydroxide.—10% (w/v) solution of potassium hydroxide (A.R.) in aqueous ethanol (3 vol. ethanol added to 1 vol. water).

Fucosterol.—Isolated from *F. vesiculosus*; m.p. 123–124° C.; $[\alpha]_D$ — 40·2° (chloroform).

Anhydrous ground seaweed (5 g. passes 64-mesh screen) is weighed into a Pyrex sintered-glass crucible (porosity 1) and continuously extracted in a Haanen and Badum extractor with boiling ether (100 ml.) for 16 hours. Any fine weed that has passed into the solvent is filtered off (G3 crucible), the ether is evaporated to dryness, and the residue dried for 1 hour at 100° C. This constitutes the crude fat.

The crude fat is refluxed with ethanolic potassium hydroxide (5 ml.) for 1 hour and the solution allowed to cool. It is then added to water (10 ml.) in a separating funnel and extracted with ether (3 × 20 ml.). The combined ether extracts are washed with water (3 × 20 ml.) and evaporated to dryness. The residue, after all traces of adhering water have been removed under vacuum over phosphorus pentoxide, is dissolved in chloroform, transferred to a 50-ml. graduated flask (a 20-ml. graduated flask is used if expected result is between 0·05% and 0·15%) and made up to mark with chloroform. An appropriate volume (10 ml. or less) of this solution is transferred to a test-tube, the volume made up to 10 ml. (if less than 10 ml. taken) with chloroform, acetic anhydride/concentrated sulphuric acid reagent (2 ml., freshly prepared) is added,

and the solution kept for ½ hour in the dark at room temperature. The solution is then transferred to a 1-cm. cell and the log I/I_0 read against a water blank using the red filter No. 608 in the Spekker photoelectric absorptiometer H.560. The amount of fucosterol is then obtained from the calibration curve obtained with pure fucosterol.

Development of method

Solvent for crude fat extraction.—Light petroleum (b.p. 40–60° c.) was used originally, but in a series of extractions carried out on *F. vesiculosus* (Feb. 1946), results varying from 1·127% to 1·265% were obtained for the crude fat content and ' channelling ' of the weed was evident. Another series was carried out on the same sample using ether as solvent, giving results of 1·834–1·842%. Ether was therefore used in the method, although it was recognized that it would remove a higher percentage of non-lipids such as pigments, etc.

Application of the Liebermann–Burchard colour reaction.—The Liebermann–Burchard reaction, which has been used extensively for the estimation of cholesterol in blood, was shown to give a green colour when applied to solutions of fucosterol in chloroform, the concentration being between 3 mg. and 5 mg. of fucosterol, which was the range expected to be suitable for seaweed examples. This reaction was therefore used as a basis for the colorimetric estimation of fucosterol.

Time taken for colour to reach maximum.—Pure fucosterol (4·4 mg.) was dissolved in chloroform (10 ml.), acetic anhydride/conc. sulphuric acid reagent (2 ml.) added and the test-tube kept in the dark at room temperature for 5 minutes when the colour started to develop. The solution was transferred to a 1-cm. cell and readings taken every 5 minutes against a water blank in the Spekker absorptiometer, using the red filter Ilford No. 608. The maximum reading was obtained after 30 minutes (timed from the addition of the acetic anhydride/conc. sulphuric acid). The colour was stable for 10 minutes, then started to fade slowly.

Calibration curve with fucosterol.—Pure fucosterol (50·6 mg.) was dissolved in chloroform (10 ml.), transferred to a 100-ml. graduated flask and made up to the mark with chloroform. This solution is equivalent to 0·506 mg. fucosterol/ml.

Standard volumes were measured into a series of test-tubes and made up to 10 ml. with chloroform where required. The colour was developed as in the method and readings taken in the Spekker absorptiometer. The results obtained are shown in Table I.

Table I

Vol. of solution, ml.	Concn. of fucosterol, mg.	log I/I_0
10	5·060	1·280
8	4·048	0·850
6	3·036	0·640
4	2·024	0·380
2	1·012	0·180

These results show a distinct linear relationship, as shown in Fig. 1.

Time required to extract the fucosterol.—In order to determine the time required to extract the fucosterol from the weed a series of three estimations was carried out on the same sample, with 8, 16 and 24 hours' extraction respectively. The results are shown in Table II.

Table II

Sample	Extraction time, hr.	% fucosterol
F. vesiculosus (Feb. 1946)	8	0·24
,, ,, ,, ,, ...	16	0·25
,, ,, ,, ,, ...	24	0·25

From Table II it would appear that 16 hours is sufficient to extract all the fucosterol, and this time was adopted in the method.

Application to seaweed samples.—Concordant results were readily obtained with this calibration curve, when applied to seaweed samples. A duplicate estimation on anhydrous milled *F. vesiculosus* (Oct. 1946) gave results of 0·26% in each case.

The validity of the method having been shown by these results, it was decided to estimate the fucosterol gravimetrically as a further check.

Estimation of the fucosterol gravimetrically as the digitonide.—The digitonin method, which

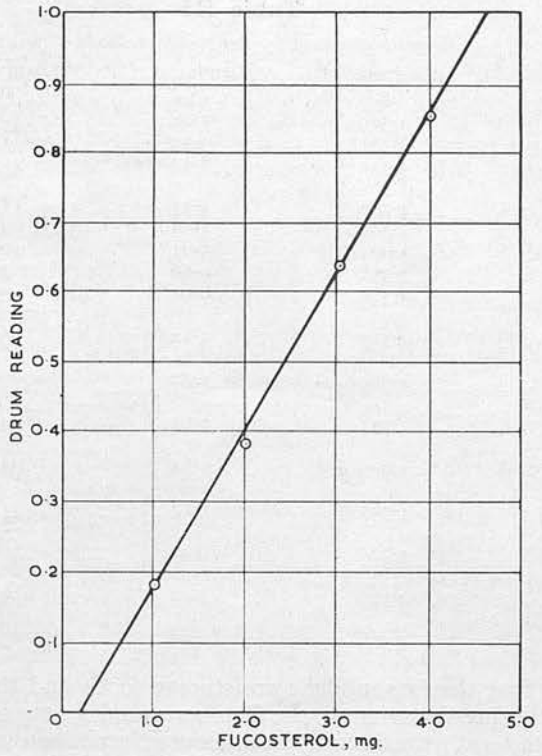

FIG. 1

has been found satisfactory for the estimation of all 3β-sterols, was employed. Anhydrous *F. vesiculosus* (Oct. 1946 ; 4·914 g.) was continuously extracted for 16 hours, the crude fat saponified, extracted with ether and evaporated to dryness as in colorimetric method. The residue, after adhering water had been removed, was dissolved in hot ethanol (10 ml.), and transferred to a small beaker ; a hot 1% solution of digitonin in 90% ethanol (6 ml.) was added, and the precipitate allowed to stand overnight in the refrigerator. The precipitate was filtered off (G3 crucible), washed with ethanol (3 × 5 ml.) and ether (2 × 5 ml.) and dried for 30 minutes at 100° C. Yield, 0·045 g.

This corresponds to 0·0123 g. fucosterol, using the formula $C_{29}H_{48}O,2H_2O$ for fucosterol (Heilbron, Phipers & Wright, 1934), and $C_{29}H_{48}O,C_{56}H_{92}O_{29}$ for fucosteryl digitonide. When calculated on the weed this is equivalent to 0·25% fucosterol, a result in good agreement with that obtained above by the colorimetric method.

A control experiment with pure fucosterol (11·5 mg.) under the same conditions gave fucosteryl digitonide (41·8 mg.) corresponding to a 99% recovery.

Recovery of fucosterol from fucosteryl digitonide.—In order to show that the results obtained were due to fucosterol and not a mixture of sterols, the digitonide prepared from the weed sample was decomposed by the method of Bergmann (1940).

Fucosteryl digitonide (28·0 mg.) was dissolved in pyridine (1·5 ml.) and kept at 90° C. for 1 hour, then evaporated to dryness under vacuum. The residue was extracted with ether (3 × 5 ml.) and the extracts combined and evaporated to dryness, leaving a wax-like solid which was dissolved in methanol (5 ml.). After evaporating to dryness, the white crystalline residue was dried under vacuum over phosphorus pentoxide. Yield 6·0 mg. M.p. 119° C. ; mixed m.p. with authentic fucosterol (m.p. 123–124° C.), 121° C. This corresponds to 78% recovery.

A control experiment with the digitonide obtained from pure fucosterol and carried out under the same conditions gave a product m.p. 120° C. corresponding to 79% recovery.

Discussion of results

The samples analysed were those collected in the vicinity of Oban during 1946 and their chemical composition has already been reported (Black, 1948, 1949).

Table III

Results obtained by proposed method

Sample		Anhydrous wt., g.	Crude fat, %	Wt. of fucosterol, mg.	Fucosterol, %
F. vesiculosus	(Feb. 1946)	4·300	1·84	10·93	0·25
,, ,,	(Oct. 1946)	4·351	3·75	11·50	0·26
Pelvetia canaliculata	(,, ,,)	4·332	8·13	12·10	0·28
Ascophyllum nodosum	(,, ,,)	4·790	4·32	11·25	0·23
F. spiralis	(,, ,,)	4·806	4·83	9·50	0·20
F. serratus	(,, ,,)	4·991	3·01	10·0	0·20
L. cloustoni frond	(,, ,,)	5·368	0·60	3·80	0·07
L. cloustoni stipe	(,, ,,)	4·585	0·61	5·50	0·12
L. digitata open sea frond	(,, ,,)	4·527	1·11	5·60	0·12
L. digitata open sea stipe	(,, ,,)	4·503	0·87	6·36	0·14
L. digitata loch frond	(,, ,,)	4·731	0·61	4·60	0·10
L. digitata loch stipe	(,, ,,)	4·639	0·63	5·90	0·13
L. saccharina open sea frond	(,, ,,)	4·547	1·17	4·20	0·09
L. saccharina open sea stipe	(,, ,,)	5·788	0·90	6·10	0·11
L. saccharina loch frond	(,, ,,)	4·599	1·22	4·10	0·09
L. saccharina loch stipe	(,, ,,)	4·526	0·93	5·70	0·13

On the assumption that the fats in algae are storage foods and that they would probably accumulate in the plant during the summer, the October samples were selected as those in which the fats, etc., were likely to be at a maximum. Samples of *F. vesiculosus* collected in February and October showed that this was so, the crude fats increasing from 1·84% in February to 3·75% in October.

The results show, in agreement with those of Russel-Wells (1932), that the percentage of crude fats decreases with depth of immersion of the weeds from 8·13% in *Pelvetia canaliculata* to less than 1% in the Laminariaceae.

A correlation exists between the percentage of fucosterol and the total crude fats, the sterol also being highest in the most exposed weeds (e.g. 0·28% in *P. canaliculata*) and decreasing to less than 0·1% in *L. cloustoni* frond.

Acknowledgments

The work described in this report forms part of the programme of research and development on seaweed undertaken by the Scottish Seaweed Research Association, and the authors are indebted to the Association for permission to publish. The authors also wish to express their thanks to Dr. Henbest, Manchester University, who provided a sample of pure fucosterol.

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Received 19 March, 1951

References

Bergmann, W. (1940). *J. biol. Chem.* **132**, 471.
Black, W. A. P. (1948). *J. Soc. chem. Ind.* **67**, 165, 355.
Black, W. A. P. (1949). *J. Soc. chem. Ind.* **68**, 183.
Carter, P. W., Heilbron, I. M. & Lythgoe, B. (1939). *Proc. roy. Soc.* [B] **128**, 82.
Coffey, D. H., Heilbron, I. M. & Spring, F. S. (1936). *J. chem. Soc.* 738.
Coffey, D. H., Heilbron, I. M., Spring, F. S. & Wright, H. R. (1935). *J. chem. Soc.* 1205.
Heilbron, I. M., Phipers, R. F. & Wright, H. R. (1934). *J. chem. Soc.* 1572.
Hey, D. H., Honeyman, J. & Peal, W. J. (1950). *J. chem. Soc.* 2881.
MacPhillamy, H. B. (1942). *J. Amer. chem. Soc.* **64**, 1732.
Russel-Wells, B. (1932). *Nature, Lond.* **129**, 654.
Sirahama, K. (1942). *J. Faculty Agric. Hokkaido Imp. Univ.* **49**, Pt. 1, 1.

Printed in Great Britain by Butler & Tanner Ltd., Frome and London

Reprinted from the Journal of the Science of Food and Agriculture, 1955, No. **1**, *pp.* **14–23**

THE PRESERVATION OF SEAWEED BY ENSILING AND BACTERICIDES

By W. A. P. BLACK

Marine algae decompose much more rapidly than land plants and various attempts have been made from time to time to preserve them. This paper mainly summarizes preliminary work carried out on ensiling from the point of view of preserving the algae for use as a possible feeding-stuff for farm animals and also for chemical processing.

Introduction

Considerable work has been carried out on the conservation of grass and forage crops and it has been found that, in order to effect their preservation with the minimum change in chemical constitution, it is essential to check respiration as soon as possible and to prevent subsequent fermentation. In many ensiling processes for land crops the carbon dioxide liberated by respiration is used to control the fermentation, and salt, sulphur dioxide, carbon disulphide, formaldehyde, formic acid, phenol, borax, benzoic acid and salicylic acid[1] have also been tried. The most practical system was ensiling, and the use of acid to lower the pH [A.I.V. (A.I. Virtanen) process] has resulted in less loss than the normal method of bacterial fermentation.

The bacterial decomposition of marine algae is a problem that has received little attention in the past, but it is at present being investigated at the Universities of Edinburgh and Nottingham. Bacteria that actively decompose several of the constituents of algae have been isolated from sea-water, from piles of decomposing weed and from different types of soil. Decomposition may take place, therefore, through the agency of organisms living in the sea or on the shore, or by infection picked up from purely terrestrial sources, or by a mixture of both.

In America, fresh *Laminaria digitata* is preserved, for later processing, by storing it in large ' curing tanks ' containing sea-water with added sodium chloride and formalin, and in Britain a patent[2] was taken out in 1937 for the preservation of algae by impregnating with sulphur dioxide gas. No attempt is made in any country, with the exception of America, to preserve fresh algae on a large scale, the normal procedure being to dry and store as dried material.

Experimental and discussion of results

The methods for the determination of water, total ash, laminarin, mannitol, cellulose, alginic acid, L-fucose, Kjeldahl and total nitrogen were those previously used by the writer ; [3–6] the water content was also determined by the Tate & Warren method[7] and the free sugars by the method of Shaffer & Somogyi.[8] The water-soluble or non-protein-nitrogen content (N.P.N.) was obtained by extracting the fresh alga (20 g.) with distilled water (150 ml.) under

boiling reflux for 30 min., filtering through filter cloth, washing the residue with hot water so that the washings and filtrate totalled 250 ml., and carrying out an estimation, as for Kjeldahl nitrogen, on 100 ml. of this solution. The N.P.N. content of the dried milled weed was determined by extracting 2 g. with 3 × 50 ml. of boiling water, refluxing in each case for 10 min. and washing the residue with 30 ml. of water. The aqueous extracts were combined, the volume was made up to 250 ml. and a Kjeldahl nitrogen estimation carried out on a 100-ml. sample. The volatile fatty acids were determined by the method of Wiegner.[9]

Effect of subdivision on the rate of decomposition

Initial experiments were carried out to determine the effect of subdivision on the rate of decomposition. The alga was either chopped into pieces or minced in a butcher's mincer. It was found that the rate of decomposition was greatly accelerated by finely dividing the weed, which produced copious exudation of mucilage.

Preservation by chemical means

Experiments were carried out with complete *Fucus serratus* plants (6–9 in. in length) immersed in sea-water containing preservatives such as potassium metabisulphite, trichlorophenol, sodium *o*-phenylphenoxide, pentachlorophenol etc. The concentration of preservative, however, required to effect preservation killed the plant, resulting in complete loss of the soluble constituents into the surrounding medium. ' Pickling ' in solutions of sea-water with at least 20% of added sodium chloride was also quite effective, but also considerably altered the composition in regard to the water-soluble constituents. Preservation in liquid media, however, involves such large volumes of liquid as practically to rule out this method as a possible means of preservation on a large scale. The spraying of the fresh algae with a solution of the preservative was not attempted because of the difficulty of ensuring, on a large scale, that the preservative permeated the entire mass. The only gaseous sterilizing agent which was tried was sulphur dioxide and this is referred to below.

Preservation by ensiling

Initial experiments were carried out on 31 October, 1951, with freshly harvested *Laminaria cloustoni* frond, *Ascophyllum nodosum* and grass. The material was finely minced and 50-g. samples were packed into 1-in.-diameter test-tubes fitted with mercury air traps and incubated at 30°, air being excluded. The results are summarized in Table I, and from these it can be concluded that *L. cloustoni* supported a more vigorous acid fermentation than the grass when made into silage. Mixtures of 80% of grass and 20% of seaweed did not reach a pH of 4·4 in seven days, which is required for satisfactory preservation; this was also found with the grass alone, which, however, appeared to have been low in sugars and unsuitable for ensiling. The bacterial counts and the other tests carried out indicated that the organisms occurring on the fresh algae, and more especially those that formed acid in the ensiled weed, were different from those found on grass and in grass silage. That, together with differences in fermentable carbohydrates, perhaps explains why the addition to grass of a relatively large amount of seaweed did not promote a more active acid formation. The bacterial plate count for *A. nodosum* showed little increase on ensiling, but that for *L. cloustoni* increased from 0·019 to 1,660 millions/g. of dry matter, which is no doubt due to the higher percentage of fermentable carbohydrates in *L. cloustoni*.

Ether extracts of aqueous homogenates were also examined on paper chromatograms with the following results:

	Lactic acid	Succinic acid	Acetic acid	Propionic acid	Butyric acid
L. cloustoni (pH 4·21)	+++	+	+++	o	+++
A. nodosum (pH 4·77)	++	+	+	+	o

After 5 weeks' incubation at 30°: Heavy spot +++ Medium spot ++ Light spot +

(1) *Laboratory-scale experiments under aerobic conditions*

Fresh *L. cloustoni* frond was minced through a ¼-in. plate and samples were packed tightly

Table I

Laboratory ensilage of grass, L. cloustoni *and* A. nodosum

	pH	Bacterial plate count, millions/g. of dry matter (D.M.)
Fresh materials		
Grass (26·5% D.M.)		60·6
Laminaria cloustoni (30·1% D.M.)	6·03	0·019
Ascophyllum nodosum (26·0% D.M.)	6·24	0·020
Ensiled 2 days at 30°		
Grass	6·23	794
L. cloustoni	6·42	151
A. nodosum	5·78	0·063
Grass + *L. cloustoni* (4 : 1)	5·76	—
Grass + *A. nodosum* (4 : 1)	5·97	—
Ensiled 3 days at 30°		
Grass	5·88	—
L. cloustoni	5·80	—
A. nodosum	5·46	—
Grass + *L. cloustoni*	5·14	—
Grass + *A. nodosum*	5·59	—
Ensiled 7 days at 30°		
Grass	5·58	191
L. cloustoni	4·20	1660
A. nodosum	4·84	0·037
Grass + *L. cloustoni*	4·84	676
Grass + *A. nodosum*	5·34	324

into 500-ml. bottles closed with ordinary parchment-paper covers, and the bottles kept under observation at 5–10°. Bottles were opened at intervals and the contents analysed. The results are given in Table II. Under aerobic conditions fairly rapid decomposition occurred, resulting in a loss of mannitol and alginic acid and a marked increase in the Kjeldahl nitrogen; laminarin appeared to be unaffected. After 80 days no mannitol or alginic acid remained. This, however, may not mean the complete breakdown of the alginic acid molecule, but only partial degradation to a molecule not precipitated by calcium chloride, and not estimated by the standard method. On the other hand, bacteria have been shown to be present in sea-water, decomposing weed and in soil which can utilize alginic acid as their sole source of carbon.[10] Recently, an alginase has been isolated from soil bacteria which hydrolyses alginic acid to mannuronic acid.[11]

Table II

Preservation of L. cloustoni *frond under aerobic conditions at* 5–10°

	Dry matter, %	Composition, % (dry basis)					
		Total ash	Laminarin	Mannitol	Kjeldahl nitrogen	Crude proteins	Alginic acid
Original sample (12/12/51)	15·1	28·0	9·2	11·0	1·91	11·9	16·7
After 22 days	17·4	31·9	8·7	4·9	2·80	17·5	5·9
,, 64 ,,	10·2	42·2	13·9	1·6	3·15	19·7	1·9
,, 80 ,,	11·6	43·0	13·0	nil	3·30	20·6	nil

(2) *Laboratory-scale experiments under anaerobic conditions*

(*a*) Fresh *L. cloustoni* frond (collected Inchcolm 12/2/52) was minced ($\frac{1}{4}$-in. plate) and packed into 500-ml. glass bottles fitted with stoppers carrying mercury seals. The bottles were maintained at temperatures of 15° and 30° under anaerobic conditions and examined at intervals. The results are given in Table III. With the material that had been treated with sulphur dioxide gas (Sample 5, Table III), no significant change in composition was evident after 50 days, the small amount of exuded cell sap at the bottom of the bottle being sufficient to account for the differences obtained. With the other samples, however, although no change occurred in the amount of dry matter, the composition had altered appreciably. The most significant change was in the content of Kjeldahl nitrogen, ' crude proteins ' appearing to be synthesized at the expense of the carbohydrates, mannitol and laminarin, as long as there

Table III

Preservation of L. cloustoni *frond under anaerobic conditions at* 15° *and* 30°

Sample	Dry matter, %	Composition, % (dry basis)									
		Total ash	Mannitol	Laminarin	Kjeldahl nitrogen (K.N.)	Non-protein-nitrogen (N.P.N.)	Protein-nitrogen (K.N. —N.P.N.)	Crude proteins (K.N. ×6·25)	Total nitrogen	Inorganic nitrogen	Alginic acid
(1) Initial sample (12/2/52) pH 7·2	17·7	31·5	9·4	6·0	2·46	1·93	0·53	15·4	3·54	1·08	14·7
(2) After 14 days at 15°	16·1	33·8	2·8	2·8	2·75	—	—	17·2	—	—	14·1
(3) After 14 days at 30° pH 6·4	16·0	34·9	1·2	1·8	3·16	2·06	1·10	19·8	3·60	0·44	13·0
(4) After 50 days at 30° pH 6·0	17·1	32·8	nil	1·2	3·24	—	—	20·3	3·56	0·32	13·2
(5) After 50 days at 30° SO_2-treated	19·2	30·9	5·3	6·4	2·60	—	—	16·3	—	—	13·5

was a supply of inorganic nitrogen. Macpherson[12] has found the determination of protein by precipitation as a copper complex to be unreliable and has found it better to extract the non-protein-nitrogen and estimate protein-nitrogen by difference. When the N.P.N., therefore, was determined for the initial sample and the sample after three days the increase was insignificant, whereas the protein-nitrogen increased from 0·53 to 1·10%, indicating that the increase in Kjeldahl nitrogen was due to true protein.

The results agree with those obtained from experiments carried out to determine the changes in composition likely to occur in the time which elapses between the harvesting of the weed and its utilization. Freshly harvested *L. digitata* plants (20) were selected at random and within 20 minutes of harvesting were treated in the following way: 2 were dried immediately, 6 were placed in a 40-gal. stainless-steel drum containing sea-water, 6 in another drum containing sea-water plus formalin (1 part in 500 parts of sea-water) and the remaining 6 were wrapped in cotton wool saturated with sea-water. The drums were covered with heavy tarpaulins to exclude light, and plants were removed at intervals, dried and analysed. With the plants in the sea-water containing the formalin, death occurred instantaneously, resulting in immediate diffusion of the mannitol and the laminarin into the surrounding sea-water. With the plants in the sea-water alone and in the cotton wool, the results were so similar that only those for the sea-water are given in Fig. 1. These results show that, in the dark, 'proteins' are synthesized at the expense of mannitol when inorganic nitrogen is available. Up to the first day, inorganic nitrogen (0·22% of dry matter) was present in the fronds, but was not detectable afterwards. During the first 24 hours the mannitol content decreased from 22·7 to 14·3%, and the crude proteins increased from 8·1 to 13·0%. The crude protein content then decreased from 13·0 to 8·8%, and the mannitol increased to 21%. At the same time the laminarin decreased from 7·7 to 1·5% and the increase in mannitol may have resulted from the breakdown of laminarin in respiration, or from the breakdown of a 'mannitol–amino-acid complex' synthesized during the first 24 hours when nitrate was available. The main differences between the plants at the beginning and the end of the test were the disappearance of inorganic nitrogen and laminarin. The results of these experiments are given as further evidence that 'protein' synthesis is possible during ensiling.

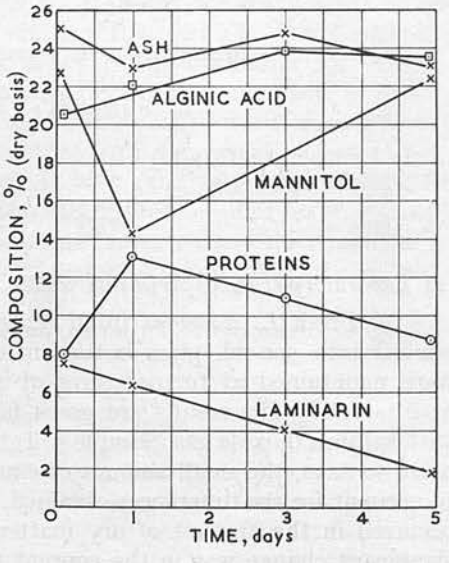

FIG. 1.—*Change in composition of* L. digitata *fronds (June* 1947), *in sea-water*

(*b*) In another series of experiments *L. cloustoni* plants collected at Inchcolm on 27 February, 1952, were separated into fronds and stipes which were minced separately through a ¼-in. plate. Samples of each were packed tightly into sterilized test-tubes (8 in. × 1½ in.), fitted with rubber bungs and mercury seals, and the tubes were maintained at 30° under anaerobic conditions. The results summarized in Table IV indicate that, with the fronds, as in the previous experiment, when inorganic nitrogen was available (1·2% of the dry matter), true proteins were synthesized whereas mannitol was utilized, the protein-nitrogen increasing from 1·43 to 2·16%. With the stipes, however, where no inorganic nitrogen was present (Kjeldahl nitrogen 1·89, total nitrogen 1·90%), no significant change in composition occurred after 72 days. Under anaerobic conditions it is interesting to note that no apparent change occurred in the alginic acid, in contrast with the breakdown which occurred under aerobic conditions.

Table IV

Preservation experiments with L. cloustoni *frond and stipe* (27/2/52) *at* 30° *under anaerobic conditions*

Frond

	Dry matter, %	Composition, % (dry basis)							
		Total ash	Mannitol	Laminarin	Kjeldahl nitrogen	N.P.N.	Protein-nitrogen	Crude proteins	Alginic acid
Initial sample	14·7	37·3	5·0	nil	2·62	1·19	1·43	16·4	15·8
After 14 days	12·5	39·5	1·1	nil	3·60	1·44	2·16	22·5	16·0
,, 72 ,,	14·4	43·1	nil	nil	4·00	—	—	25·0	16·2

Stipe

	Dry matter, %	Composition, % (dry basis)				
		Total ash	Mannitol	Laminarin	Crude proteins	Alginic acid
Initial sample	15·4	38·8	6·2	nil	11·7	19·3
After 14 days	15·9	37·0	4·5	nil	11·8	19·4
,, 72 ,,	14·0	37·8	4·0	nil	11·5	19·2

(*c*) *L. digitata*, *L. cloustoni* and *L. saccharina* fronds collected at Inchcolm on 16 June, 1952, were minced through a ¼-in. plate and samples packed into six sterilized test-tubes (8 in. × 1½ in.), fitted with rubber stoppers and mercury seals. The tubes were incubated at 30°, two were removed at intervals of 24, 48 and 72 hours, and the contents dried and analysed. The analytical results are given in Table V ; they agree with those previously obtained. Only where there was inorganic nitrogen present in any quantity was there any major change in chemical composition. With *L. saccharina* low in inorganic nitrogen, no significant change in composition occurred. In contrast, *L. digitata* contained a reserve of inorganic nitrogen and the mannitol was almost completely utilized, and there was an increase in the crude protein content from 11·5 to 14%. *L. cloustoni* was intermediate between *L. digitata* and *L. saccharina*, the mannitol content decreasing from 9·1 to 5·1% and the crude protein increasing from 11·5 to 13% (dry basis). In all cases, the seaweed constituents exerted a buffering action and prevented the pH from falling below 4·7.

Pilot-scale experiments

The experiments were carried out in concrete pipes, coated internally with bitumen paint (Fig. 2), which held approximately 6 cwt. of chopped weed. Except with *L. cloustoni*, when two days elapsed between harvesting and ensiling, the algae, the same day as harvested, were put through a Robust Cutter, giving pieces 1 in. and less, and packed tightly into the pipes until 1 ft. from the top. This was covered with bitumen paper, and soil was added to the last foot of pipe and pressed firmly down. A metal cover was then put over the top to exclude rain. Samples of liquor were withdrawn at intervals from the run-off pipe. Samples of the algae were also packed into 500-ml. glass bottles fitted with stoppers and mercury seals and incubated at 30°.

(1) A. nodosum *collected at South Queensferry* 4/7/52.—The results of this experiment are summarized in Table VI. As a check on the oven-drying method, the water in the fresh samples after ensiling was also estimated by the Tate & Warren method ;[7] both methods gave almost

Table V

Preservation experiments with L. digitata, L. cloustoni *and* L. saccharina *fronds collected Inchcolm 16/6/52 and incubated at 30° ; laminarin was absent from all samples*

	L. digitata frond	*L. cloustoni* frond	*L. saccharina* frond
pH			
Initial	6·80	6·60	6·52
After 24 h. incubation	7·05	6·12	6·26
,, 48 ,, ,,	5·73	5·70	5·70
,, 72 ,, ,,	5·03	4·71	4·80
Water content, %			
Initial	87·7	86·7	88·3
After 24 h. incubation	88·0	86·8	87·4
,, 48 ,, ,,	87·6	89·1	86·7
,, 72 ,, ,,	89·0	88·2	87·0
Ash, % (dry basis)			
Initial	40·8	37·0	35·9
After 24 h. incubation	41·1	37·6	35·5
,, 48 ,, ,,	39·5	35·4	34·4
,, 72 ,, ,,	43·5	40·4	35·5
Mannitol, % (dry basis)			
Initial	10·5	9·1	14·8
After 24 h. incubation	12·7	9·5	16·1
,, 48 ,, ,,	9·6	8·7	15·2
,, 72 ,, ,,	2·1	5·1	13·7
Total nitrogen, % (dry basis)			
Initial	2·27	2·00	2·09
Kjeldahl nitrogen, % (dry basis)			
Initial	1·84	1·84	1·93
After 24 h. incubation	2·10	1·79	2·09
,, 48 ,, ,,	2·02	1·80	1·92
,, 72 ,, ,,	2·25	2·07	2·00

identical results. This proved that the loss on drying at 100–105° was water and not volatile matter etc. formed during ensiling.

The *A. nodosum* contained very little inorganic nitrate and, with the exception of a slight increase in the Kjeldahl and the water-soluble nitrogen, no significant changes in composition occurred after incubating or ensiling in the pipe for 102 days. The percentage of soluble nitrogen increased from 24% of the Kjeldahl nitrogen in the initial sample to 30% after 102 days in the pipe, representing 8% protein breakdown, which is considerably less than is found for grass on ensiling. The water content before and after incubating was 66·8 and 67·1% respectively, and the differences in the ash, laminarin and mannitol contents were also insignificant. When the silo was opened after 102 days, except for a trace of mould on the surface of the algae which had been in contact with the soil at the edges, the algae appeared to be unchanged and looked as ' fresh ' as when put into the silo. Samples were taken at each foot depth and, with the exception of differences in pH (4·94 at the top increasing to 5·31 midway down and then decreasing to 4·86 at the bottom of the silo), the differences in composition were insignificant. The sample from the bottom of the silo was analysed for acetic and lactic acids and the results of 0·45 and 1·25% respectively (calculated on the fresh weight) are of the

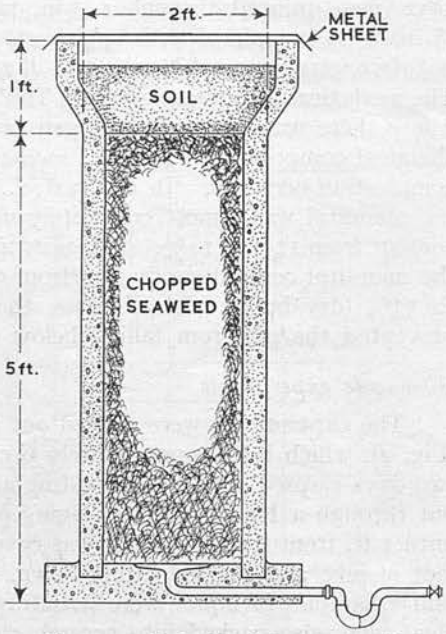

FIG. 2.—*Experimental concrete silo*

Table VI

Ensiling experiment with A. nodosum *collected South Queensferry 4/7/52*

	pH	Water content, %		Composition, % (dry basis)					
		Oven method	Tate & Warren[7]	Total ash	Laminarin	Mannitol	Cellulose	Alginic acid	L-Fucose
Initial sample	6·26	66·8	67·0	19·6	6·2	8·8	4·0	24·3	7·3
After 48 h. incubation at 30°	5·38	66·8	66·3	19·0	6·0	9·7	3·8	24·2	—
After 72 h. incubation	5·00	66·5	65·7	18·6	6·1	10·2	2·3	24·1	—
After 96 h. incubation	5·29	67·0	65·4	18·7	5·3	10·0	2·3	23·2	—
After 120 h. incubation	5·47	67·1	66·8	19·0	4·8	9·4	2·1	23·1	—
After 21 days' incubation	5·43	66·3	65·6	18·8	5·4	10·2	2·5	24·2	7·1
After 102 days' incubation	5·47	67·1	—	19·6	5·3	10·5	1·7	24·0	—
After 102 days in pipe, 0–1 ft.	4·94	67·6	—	20·2	4·5	10·1	1·9	22·3	—
After 102 days in pipe, 1–2 ft.	5·10	67·3	—	19·7	4·3	10·1	1·7	—	—
After 102 days in pipe, 2–3 ft.	5·31	67·7	—	19·8	4·2	9·8	1·6	—	—
After 102 days in pipe, 3–4 ft.	5·11	67·5	—	19·8	3·9	9·2	2·8	24·7	—
After 102 days in pipe, 4–5 ft.	4·86	67·1	—	19·6	4·5	8·4	2·5	24·2	7·4

	Fresh weed			Dried milled weed		
	Kjeldahl nitrogen	Total nitrogen	N.P.N.	Kjeldahl nitrogen	Total nitrogen	N.P.N.
Initial sample	1·36	1·44	0·33	1·44	1·54	0·47
After 48 h. incubation at 30°	1·38	—	0·32	1·45	1·52	0·42
After 72 h. incubation	1·40	1·47	0·37	1·42	1·48	0·51
After 96 h. incubation	1·47	1·47	0·42	1·40	1·45	0·54
After 120 h. incubation	1·41	1·48	0·40	1·47	1·52	0·55
After 21 days' incubation	1·39	1·42	0·38	1·41	1·44	0·45
After 102 days' incubation	1·50	1·55	0·42	1·52	1·55	0·61
After 102 days in pipe, 0–1 ft.	1·51	1·56	0·46	1·51	1·54	0·76
After 102 days in pipe, 1–2 ft.	1·54	1·55	0·46	1·49	1·52	0·68
After 102 days in pipe, 2–3 ft.	1·50	1·55	0·46	1·49	1·55	0·68
After 102 days in pipe, 3–4 ft.	1·55	1·58	0·47	1·55	1·58	0·69
After 102 days in pipe, 4–5 ft.	1·50	1·57	0·44	1·60	1·62	0·67

same order as normally found in ensiled grass. A negative result (— 0·9%) was obtained for butyric acid.

Samples of liquor were drained from the silo at intervals and their analytical figures are given in Table VII. The total weight obtained was 10,540 g., representing 3·1% of the weed in the silo (wet weight). The liquor contained the water-soluble constituents, was particularly high in mannitol (20% of the dry matter) and contained 9·6% of crude proteins (dry basis).

(2) A second ensiling experiment was carried out with *A. nodosum* (6 cwt.) collected at South Queensferry in December, 1952, and the results are given in Table VIII. Before opening the silo, 107 lb. of liquor, representing 16% of the initial fresh weight of the algae, was drained off. As in the previous experiment, preservation occurred with very minor changes in chemical composition, which could be accounted for by the losses in the cell sap drained from the silo.

In the previous experiment, partial drying of the weed occurred on the shore after collection which, no doubt, accounted for the smaller amount of liquor drained from the silo.

(3) L. cloustoni *collected at Loch Feochan, Argyllshire, 7/1/53.*—The *L. cloustoni* (whole plants) was collected at Loch Feochan on 7 January, 1953, and was chopped up and filled into the silo on 9 January. It is probable that some change in composition may have occurred

Table VII

Analysis of liquor drained from* A. nodosum *silo of 4/7/52*

Date	pH	Water content	Total ash	Laminarin	Mannitol	Alginic acid	L-Fucose	Kjeldahl nitrogen
11/7/52, 7 days after closing silo	4·84	84·6	30·6	—	20·3	—	—	—
17/7/52	4·78	85·0	28·7	—	18·9	—	—	—
25/7/52	4·80	84·6	34·5	8·0	17·4	0·6	3·3	1·53
15/10/52	5·06	83·6	32·2	—	7·4	—	—	—

* With the exception of the water content the results are expressed as a percentage of the dried residue.

during this time. At the same time samples were placed in bottles with stoppers fitted with mercury seals and incubated at 30°. The silo was opened up after 60 days and the analytical results of the samples from this experiment are given in Table IX. The material in the silo appeared 'fresh' and unchanged. Analysis, however, showed that there had been some utilization of the laminarin (13·4 to 2·7%) accompanied by a decrease in the mannitol content (13·9 to 8·7%), but with no significant change in the crude proteins. There was an increase in the water-extractable nitrogen, from 0·48 to 1·0%, that is an increase in the percentage of soluble nitrogen from 26 to 55%, representing protein breakdown equal to 39%, which is considerably greater than that found for *A. nodosum* (cf. 8%). Free sugars (estimated as glucose) were found to the extent of 1·46% (dry basis) in the ensiled algae, and the lactic acid content, 2·04% (fresh weight), was again of the same order as that of ensiled grass. The grade of the alginic acid appeared to be unaffected.

From the 737 lb. of chopped weed filled into the silo, 74 lb. of liquor was drained off (10% of total weed) and 630 lb. (86% recovery) was obtained from the silo, accounting for 96% of the alga. The dry material from this liquor was high in ash (56·6%) and mannitol (16·3%) and contained 6·1% of crude proteins and about 5% of fucoidin.

This last experiment with *L. cloustoni* was repeated, but a depth of only 3 ft. of chopped plant was placed in the silo without any soil to exclude air, and a loose cover placed over the top to prevent access of rain. The *L. cloustoni* (whole plants) was collected at Inchcolm on 12 December, 1952, chopped up the following day and packed into the silo. After 97 days the material was examined. The top 6 in. appeared to be in an advanced state of decomposition and smelt strongly of ammonia. Analysis of samples gave the results shown in Table X. These figures would appear to indicate that some preservation can be effected without sealing off the silo with soil, but only at the expense of a certain amount of decomposition in the upper layers.

Summary of results

Experiments on the preservation of the brown marine algae are described. The chemical methods studied include the immersion of the algae in solutions containing bactericides and the treatment of the fresh algae with sulphur dioxide gas.

It is possible to preserve the algae in sea-water containing more than 20% of sodium chloride, but not without major chemical changes in composition. Plasmolysis results in a loss of the soluble constituents into the surrounding aqueous medium and an increase in the ash content of the plant.

The addition of bactericides appears to be effective only at a concentration that 'kills' the plant, which again results in a loss of the soluble constituents. Spraying of the algae with bactericides was not attempted because of the difficulty of ensuring intimate contact. Treatment of the fresh algae with sulphur dioxide gas, followed by exclusion of air, is very effective and very little change in chemical composition occurs.

Table VIII

Ensiling experiment with* A. nodosum *collected at South Queensferry* 3/12/52

Sample	pH	Water content	Total ash	Laminarin	Mannitol	Cellulose	Alginic acid	L-Fucose	Wet weed			Dried milled weed		
									Kjeldahl nitrogen	Total nitrogen	N.P.N.	Kjeldahl nitrogen	Total nitrogen	N.P.N.
Initial	6·52	73·3	22·3	2·6	8·7	2·4	23·3	6·9	1·76	1·82	0·56	2·00	2·07	0·66
After 48 h. incubation at 30°	5·62	72·9	21·3	2·3	8·5	2·3	—	6·0	1·60	1·50	0·63	2·02	2·08	—
After 67 days' incubation at 30°	5·02	75·2	22·7	1·9	7·5	—	21·2	7·1	1·59	1·69	0·50	1·82	1·93	0·35
After 67 days in pipe	4·78	70·4	21·7	2·8	5·7	—	20·6	8·0	1·86	1·99	0·51	1·84	1·77	0·49
Liquor drained from pipe before emptying	4·76	91·5	44·8	2·9	16·9	—	—	1·8	—	—	—	2·03	2·34	—

* With the exception of the water content the results are expressed as a percentage of the dry matter

Table IX

Ensiling experiment with* L. cloustoni (*whole plant*) *collected at Feochan* 7/1/53

Sample	pH	Water content	Total ash	Laminarin	Mannitol	Alginic acid	L-Fucose	Wet weed			Dried milled weed			Wet weed			Viscosity of 0·25% (w/w) soln. of sodium alginate, cs	Free sugars as glucose
								Kjeldahl nitrogen	Total nitrogen	N.P.N.	Kjeldahl nitrogen	Total nitrogen	N.P.N.	Acetic acid	Butyric acid	Lactic acid		
Initial	6·72	82·3	34·8	13·4	13·9	14·2	3·5	1·85	1·76	0·48	1·89	2·02	0·73	—	—	—	13·9	—
After 48 h. incubation at 30°	5·87	82·4	34·8	7·2	12·1	11·4	3·5	1·98	1·90	0·64	1·80	1·87	0·71	—	—	—	—	—
After 60 days' incubation	4·77	84·4	37·5	1·4	2·2	12·6	3·5	2·11	1·96	1·04	2·10	2·24	0·45	—	—	—	—	—
After 60 days in pipe	5·43	81·0	33·3	2·7	8·7	16·2	4·0	1·80	1·73	0·99	1·72	1·88	0·64	0·87	−0·39	2·04	11·5	1·46
Liquor drained from pipe before emptying	5·16	89·3	56·6	nil	16·3	—	2·9	—	—	—	0·98	1·05	—	—	—	—	—	—

* With the exception of the water, acetic, butyric and lactic acid contents, the results are all expressed as a percentage of the dry matter

Table X

Sample		pH	Water content, %	Dry basis: Mannitol, %	Dry basis: Ash, %	Dry basis: Kjeldahl nitrogen, %
Original		6·70	84·0	10·1	34·7	2·65
After 97 days	Top 6 in.	7·32	87·3	1·5	44·2	3·16
	Remaining 2½ ft.	5·09	84·1	2·0	27·7	3·15
	Liquor drained off	5·04	92·9	1·2	60·4	1·89

The ensiling of algae has been studied both on the laboratory and on the pilot scale and it has been found that they support a vigorous lactic acid fermentation and can be ensiled without inoculation or the addition of fermentable carbohydrates.

The chemical changes that occur in algae on ensiling depend on the initial composition of the material, and the extent of the change, therefore, depends on the time of year at which the algae are harvested. With *A. nodosum* very little change in composition occurs with season, and preservation can be accomplished by ensiling, the only significant change being an increase in non-protein-nitrogen at the expense of the protein-nitrogen. With *L. cloustoni*, marked seasonal changes occur in the chemical composition. If harvested in the spring of the year when the plant contains inorganic nitrogen, and laminarin is absent, ensiling results in protein synthesis with utilization of the mannitol. If harvested later in the year, when inorganic nitrogen is absent and laminarin is present, fermentation results in the utilization of the laminarin and a breakdown of proteins. Consequently, the preservation of *A. nodosum* can be accomplished with considerably less change in chemical composition than *L. cloustoni*.

It is reasonable to assume that similar results would be obtained if the size of the silo is increased from 6 cwt. to 20 tons. Recent work at the Edinburgh and East of Scotland College of Agriculture (private communication) has shown that the results obtained with grass in concrete silo pipes, of a capacity approximately equal to that used in the present investigation, are similar to those obtained in silos of the order of 20 tons.

One of the main objectives of this work has not been fulfilled. It was intended to carry out digestibility trials with sheep at the Edinburgh and East of Scotland College of Agriculture, but, unfortunately, the breed of sheep available would not accept the ensiled algae.

Acknowledgments

This work forms part of a programme of research and development undertaken by the Institute of Seaweed Research, and the author is indebted to the Institute for permission to publish. He wishes to thank Professor S. J. Watson and the staff of the Edinburgh and East of Scotland College of Agriculture for their valuable advice, and for carrying out the preliminary experiments. Thanks are also due to Mr. W. J. Cornhill and Mr. J. C. Paterson for assistance with the analytical work.

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Received 20 May, 1954; (amended manuscript) 3 September, 1954

References

[1] Watson, S. J., 'The Science and Practice of Conservation—Grass and Forage Crops', 1939, Vol. I (London: Fertilisers and Feeding-stuffs Journal)
[2] Bonniksen, C. W., B.P. 473,885
[3] Black, W. A. P., *J. Soc. chem. Ind., Lond.*, 1948, **67**, 165
[4] Black, W. A. P., Cornhill, W. J., Dewar, E. T., Percival, E. G. V., & Ross, A. G., *J. Soc. chem. Ind., Lond.*, 1950, **69**, 317
[5] Black, W. A. P., & Dewar, E. T., *J. Mar. biol. Ass. U.K.*, 1949, **28**, 673
[6] Black, W. A. P., *J. Mar. biol. Ass. U.K.*, 1950, **29**, 379
[7] Tate, F. G. H., & Warren, L. A., *Analyst*, 1936, **61**, 367
[8] Shaffer, P. A., & Somogyi, M., *J. biol. Chem.*, 1933, **100**, 695
[9] Wiegner, G., 'Anleitung zum quantitativen agrikulturchemischen Praktikum', 1926, p. 254 (Berlin: Borntraeger)
[10] Chesters, C. G. C., unpublished
[11] Kooiman, P., *Biochim. biophys. Acta*, 1954, **13**, 338
[12] Macpherson, H. T., *J. Sci. Fd Agric.*, 1952, **3**, 362

Printed in Great Britain by Butler & Tanner Ltd., Frome and London